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3)***Mechanical detection of interaction of small specific ligands with
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Morozov V N; Morozova TYa
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Mechanical Detection of Interaction of Small Specific Ligands with Proteins and DNA in Cross-Linked Samples

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Cross-linked crystalline and amorphous films of different proteins and cross-linked DNA gels were found to change their mechanical properties when soaked in solutions of specific ligands at nearly physiological concentrations. This chemomechanical effect may be used to rapidly (within a few minutes) detect the ability of macromolecules to bind small (less than 1 kDa) ligand molecules, to measure concentrations of ligands (higher than 10 nM), and to estimate binding constants (lower than 10^7 M^{-1}). Only 0.1–1 mg of protein or DNA is needed to prepare more than 10 samples sufficient for a large number of tests, provided binding is reversible. The method is recommended for rapid primary screening in search of new drugs, in biochemical studies, and as a basis for designing biosensors and other analytical instruments.

Studies of the interaction of proteins and DNA with small ligands are commonplace in biochemical, pharmacologic, and toxicological research. To detect the interaction of macromolecules with their specific ligands in solution, one should either measure binding-induced changes in physical properties of the macromolecule or of the ligand (optical, NMR, etc.) or use some partitioning technique (equilibrium dialysis, size-exclusion chromatography, etc.). Both of these approaches have some serious limitations. For example, the optical properties of a macromolecule will change only if some reporter group (fluorescent or absorbent) is by chance situated near the binding site. On the other hand, partitioning methods require the determination of small concentrations of ligands, which often necessitates synthetic attachment of radioactive or chromogenic labels. This makes the procedure expensive, tedious, and time consuming. Some of these negative features have been avoided recently by the use of titration calorimetry (1). Nevertheless, in research on a large series of ligands, considerable consumption of protein and DNA in solution may be a disadvantage to all these methods.

Here, we propose a new method for detecting the interaction of macromolecules with their specific ligands by exploiting the ability of cross-linked protein and DNA solid and gel samples to change their mechanical properties upon binding of the specific ligands with the macromolecules. This chemomechanical phenomenon was initially discovered in lysozyme crystals (2,3) and in papain films (4). We suggest that the phenomenon has some universal character because it is based on two well-known facts. First, many crystalline proteins retain their ability to bind specific ligands. This fact is widely used in the X-ray analysis of protein–ligand complexes (5). Second, whatever the character of this binding, to some extent it changes inter- and intramolecular interactions, the molecular structure, and the packing of molecules in the solid sample. Of all the physical parameters, the mechanical ones seem to be the most sensitive to these changes. The theoretical analysis presented in the Appendix shows that deformation of protein molecules by 10^{-6} Å can be, in principle, measured with mechanical methods. In practice, changes in average protein dimensions of 10^{-2} Å can be readily detected. This work was performed to test the hypothesis of a universal character of chemomechanical interactions in protein and DNA solids and gels.

The results presented here demonstrate that the chemomechanical effect is inherent in nearly all solids of different proteins and in DNA gels tested, thus offering a number of potential applications such as the design of new types of chemical sensors, chromatography detectors, and screening ligands that specifically bind protein and DNA molecules.

MATERIALS AND METHODS

Materials. The protein and DNA preparations used are specified in the footnotes to Table 1. All other reagents were of analytical grade or higher quality.

Protein film preparation. The preparation is shown schematically in Fig. 1. A salt-free protein solution (10–

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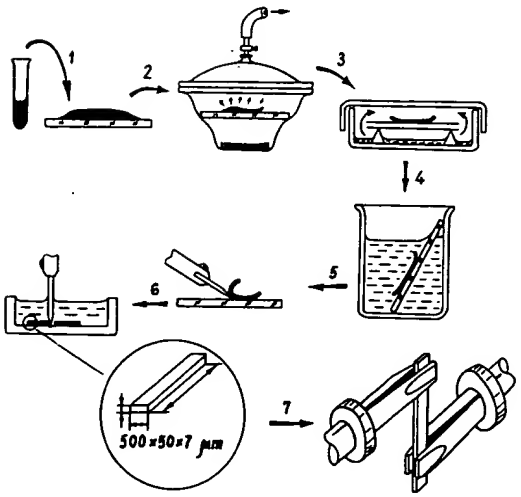

FIG. 1. The preparation of cross-linked amorphous protein samples. *1*, Pouring of protein solution onto a glass surface; *2*, rapid drying under reduced pressure; *3*, cross-linking in a vapor of glutaraldehyde; *4*, washing; *5*, detachment of the film; *6*, cutting of samples; *7*, attachment to micropincers in the device.

200 mg/ml) was poured onto a glass plate and rapidly dried under reduced atmospheric pressure on evacuation (10–15 mm Hg). Dry protein film (5–10 μm thick) was then cross-linked in a vapor of 25% glutaraldehyde (GA)[1] solution at 25–27°C. The time required to obtain unsoluble film varied between 0.5 and 6 h for different proteins. Cross-linked film was washed with water, to remove the excess aldehyde, and then carefully detached from the glass plate. We refer to this as the standard procedure; some modifications of the standard procedure are specified in the footnotes to Table 1.

Films were stored mainly under two conditions: in a refrigerator at 4°C in a buffer solution, used to check specific binding, or in a dried form at −15°C.

DNA film preparation. A calf thymus DNA precipitate in alcohol was dialyzed against 10 mM NaCl solution to give a gel with a DNA concentration of about 100 mg/ml. The gel was then distributed over the glass surface, dried as described for protein films, and cross-linked by uv irradiation under a germicidal lamp (30 W, 0.1 m to sample) for 1.5 h (6).

Growth and cross-linking of protein crystals. Crystal growth and cross-linking are described in the footnotes to Table 1.

Sample preparation. Before the measurement, a strip 300–700 μm long and 20–50 μm wide was cut with a razor blade from films or microtome sections of protein crystals, as described in (7).

Apparatus. The mechanical part of the instrument used to measure Young's modulus and isometric stress in protein and DNA samples is shown schematically in Fig. 2. The sample (*7*) is attached to two micropincettes (*5,8*) made from a split tungsten wire as described in Ref. (7). Pincers (*5*) are attached to a force transducer. The force transducer consists of a quartz cantilever beam (*3*) (a plate 0.8 × 3 × 15 mm) with a vacuum-deposited gold coating, making a measuring capacitance with another gold-coated quartz plate (*4*). Bending of the quartz plate under the action of the force applied to the sample resulted in the capacitance changes measured with the capacitometer. Another micropincette (*8*) is attached to a bimorph (*9*), whose bending upon application of voltage is controlled through the changes in capacitance between the bimorph surface and the gold-covered quartz electrode on the plate (*10*). The sample deformation block is made on a quartz plate (*10*) capable of rotating around a quartz rod (*14*) with guides (*13*) when the screw (*12*) position is changed. This enables the distance between the ends of the pincettes in the range 0–700 μm to be changed with a precision of about 1 μm. Both the force transducer and the deformation block are covered with plastic housings (not shown in Fig. 2), protecting them from dust and humid air.

The sample is placed in a flow chamber (*6*) with a volume of 33 μl. The pincettes penetrate the chamber via L-like slots. The buffer solution enters the chamber

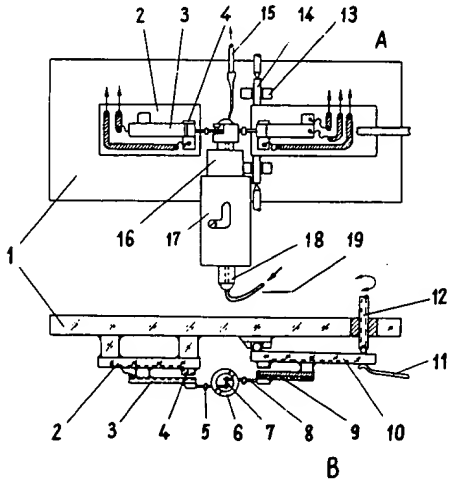

FIG. 2. Schematic view of the mechanical part of the device. (A) Front view; (B) top view. *1*, General bedplate; *2*, base of force transducer; *3*, force measuring the cantilever beam; *4*, gold-coated quartz electrode; *5*, pincers; *6*, flow chamber; *7*, sample; *8*, pincers; *9*, bimorph; *10*, base plate of the deformation unit; *11*, spring; *12*, screw; *13*, guides; *14*, axis; *15*, suction capillary; *16*, chamber holder; *17*, holder guide; *18*, glass capillary; *19*, solution inlet. Details *1–4*, *10*, *13*, and *14* are made of a fused quartz connected with epoxy glue. Arrows indicate electric connections.

[1] Abbreviations used: GA, glutaraldehyde; GlcNAc, *N*-acetyl-D-glucosamine; 2′(3′)-CMP, cytidine-2′(3′)-monophosphate; Hipp-Phe-L, Hippuryl-DL-phenyllactate; Phe, DL-phenylalanine; BAEE, *N*α-benzoyl-L-arginine ethyl ester; BAA, *N*α-benzoyl-L-arginin-amide; TNPS-Gly, 2,4,6-trinitrophenyl-1-sulfonylglycine; 2,4-DNP, 2,4-dinitrophenol; 2,5-DNP, 2,5-dinitrophenol; CrP, creatine phosphate; TNT, 2,4,6-trinitrotoluene; BSA, bovine serum albumine.

through the thick-wall glass capillary (*18*) and is continuously sucked out through a small plastic tube (*15*) placed over the chamber. The tube is attached to a water pump.

The capacitometer of both the force transducer and the deformation transducer consisted of battery-powered autogenerators (with frequencies of 30 and 25 MHz, respectively) made on reversed diodes according to the method of Boghosian *et al.* (8). The scheme was chosen for its low voltage in the capacitor, stability, and extremely low energy consumption. The signals from the generators were mixed with those from standard quartz-stabilized generators, and the difference frequency (in the range 0–500 kHz) was extracted and displayed in a digital form. A frequency-to-voltage transducer allowed force and deformation signals to be continuously recorded in the range 0.5–15 kHz (deviation from linearity was less than 1%).

Calibration procedure and performance parameters. The sensitivity of the force transducer measured by hanging pieces of a steel wire of definite weights was $(2.04 \pm 0.03) \times 10^8$ Hz/N or $(1.46 \pm 0.02) \times 10^5$ V/N. Deviation from a linear response in the operating range (forces $<5 \times 10^{-4}$ N) did not exceed 1%. The instability of the output of the force transducer with free pincette was less than 3×10^{-7} N in 10 min. Compliance of the force transducer and bimorph, measured with Linnik's interferometer, was $s_f = 1.7 \times 10^{-3}$ m/N and $s_d = 4.3 \times 10^{-4}$ m/N, respectively. Resonance frequencies of both the force transducer and the bimorph were higher than 15 Hz.

Measured characteristics. The device enables static tensile deformation to be applied, $\epsilon_0 = (L - L_0)/L_0$ (L_0 is the initial length of the sample; L is the length of the deformed sample, maintained constant in isometric measurements), and isometric tension, F, generated in the sample as a result of the deformation, to be measured. Another measured mechanical parameter of the sample is its compliance, which is determined as a relation of the amplitude of small harmonic deformations of the sample, $\Delta L \ll (L - L_0)$, to the amplitude of the deformation-induced tension oscillations, ΔF:

$$\kappa = \Delta L/\Delta F. \qquad [1]$$

Measured values of F and κ are conveniently expressed as isometric stress σ and Young's modulus E, which characterize the material of the sample, independent of its dimensions. They are defined by the relations

$$\sigma = F/bh \qquad [2]$$

and

$$E = \kappa^{-1}L_0/bh. \qquad [3]$$

Here, b and h are the width and thickness of the sample. Relations [1]–[3] are applicable only when the compliance of the sample is much higher than the sum of the compliances of the force transducer and the block of sample deformation; i.e., $\kappa \gg s_f + s_d$. This was satisfied in all our samples.

Ligand-induced deformation of the sample, $\epsilon_L = (L_1 - L_0)/L_0$ (L_1 is the length of the sample in ligand solution with no tension applied) can be calculated from measured changes in isometric tension, ΔF, and in compliance, $\Delta\kappa$, according to the tension–deformation relation in a simple spring:

$$\epsilon_L = -\epsilon_0[(\Delta\kappa/\kappa) + (\Delta F/F)]. \qquad [4]$$

Measurement procedure. Before measurement the sample is clamped in micropincettes in the device by the use of a micromanipulator and stretched by $\epsilon_0 = 1.5$–4% to achieve the maximum Young's modulus value. In measurements of Young's modulus, the sample is periodically stretched with frequency 0.1–0.15 Hz and with amplitude, $\Delta L/L_0$, usually not exceeding 0.1%. After the relaxation process was complete (15–30 min) and a steady isometric tension set, F, the flux of the buffer, was changed for each solution of ligand and changes in F and in κ were registered. To decrease the consumption of ligands, in some cases they were added as concentrated solutions into the chamber filled with stirred buffer with no flow. To maintain a constant droplet volume, in this case a special electronically controlled system added water as it evaporated.

RESULTS

The results of tests of 16 different proteins and DNA samples are presented in Figs. 3–7 and summarized in Table 1. Binding of specific ligands was found to affect the mechanical parameters of nearly all the protein and DNA samples studied. Thus, of 35 specific ligands tested, only 2 (lactic and pyruvic acids with lactate dehydrogenase) had no effect when taken at physiological concentration. Most of the ligands induced a 5–40% change in isometric stress in samples made of their specific target macromolecules; however, in some cases the effect exceeded 100%. Compliance or Young's modulus of samples usually changes considerably less (1–10%) and frequently does not change at all. In some protein samples, however, this parameter changed considerably. Thus, the modulus of parvalbumin films decreased from 100 to 20 MPa upon their transfer from 1 mM Ca solution to EDTA solution. Ligand-induced deformation, ϵ_L, varied between 0.01 and 4% in different samples.

The same substance may produce quite different effects on different samples. Thus, binding of glucose to hexokinase film resulted in an increase of isometric tension, whereas concanavalin film responded by a decrease in the tension on the glucose solution. In the con-

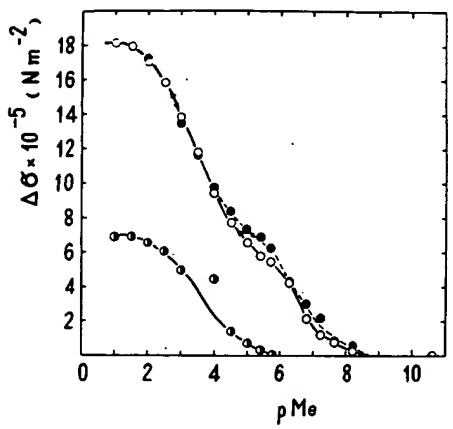

FIG. 3. Changes in isometric stress in carp parvalbumin film in solutions with different concentrations of free Ca ions (empty circles, on decrease of pCa; filled circles, on subsequent increase of pCa) and Mg ions (half-filled circles). For experimental conditions see the footnotes to Table 1. pMe denotes -log[Me], and [Me] is the concentration of free metal ions in solution.

trast, the same protein film may respond differently on different specific ligands: substrate, glucose, and competitive inhibitor, *N*-acetyl-D-glucosamine, produced changes in the tension of opposite sign in hexokinase film.

The time needed to reach an equilibrium in the binding of ligands with protein molecules within films is strongly dependent on the size of the ligand molecules and their concentration as well as on the thickness of the film. With ligands of 100–300 Da the equilibrium was reached in 3–5 min in the millimolar concentration range if the films do not exceed 5–7 μm in thickness. Equilibration in solutions with concentrations lower than 10^{-7} M usually requires more than half an hour, making equilibrium measurements unreliable and tedious.

In all studied combinations of ligand–protein in which equilibrium is reached at every concentration, the concentration dependence of changes in isometric tension looks like an adsorption isotherm with one or a few binding centers, as can be seen in Fig. 3 for parvalbumin. Plotted with double-reciprocal coordinates, the concentration dependence is linearized to give one or two binding constants. An example of such a plot of the data for concanavalin is presented in Fig. 4. A comparison of the dissociation constants found with those published in the literature for the same proteins in solutions is presented in Table 1. Usually the difference between the constants does not exceed an order of magnitude.

Binding of the ligands with low affinities (dissociation constant, $K_d \geq 10^{-6}$ M) was found to result in completely reversible changes in the mechanical properties of samples: 5–30 min of washing samples in buffer solution was enough to restore initial tension, compliance, and sensitivity to their ligand.

Desorption of ligands from samples with high affinity to the ligand requires a long time and makes their chemomechanical effects practically irreversible. Thus, films of monoclonal antibodies against TNPS-Gly ($K_d \cong 10^{-8}$ M) restored their sensitivity to low hapten concentrations only after 2 days of washing in buffer solution. Biotin with $K_d = 10^{-15}$ M caused a large but completely irreversible decrease in tension in avidin films.

Washing of strongly bound ligands can be greatly accelerated in the presence of molecules complexing the ligand and capable of penetrating the sample. Rapid washing of Ca ions from parvalbumin film with EDTA solution, seen in Fig. 5, demonstrates the phenomenon and makes possible rapid measurements of concentration of the ligand with high affinity in equilibrium mode. Comparison of isometric tension in a parvalbumin film in a buffer solution and in control EDTA–Ca buffers enables measurements of pCa with an accuracy of 0.1 unit.

Another way to rapidly detect the specific ligands with high affinity and to measure their concentration is to record the first derivative of the signal. As shown in Fig. 6, the concentration dependence of the derivative plotted in logarithmic scale is linear over a wide range of concentrations. The lowest concentration detectable with such a method is about 10^{-8} M.

Not only protein but also DNA films show considerable changes in mechanical properties after binding specific ligands, as illustrated in Fig. 7. It can be seen that ethidium bromide reveals a concentration dependence different from that of acridine orange and actinomycin D. The latter two intercalators show a marked cooperativity of binding, whereas the effect of ethidium changes its charge at high concentrations.

DISCUSSION

The data presented under Results provide convincing evidence that the chemomechanical effect has a univer-

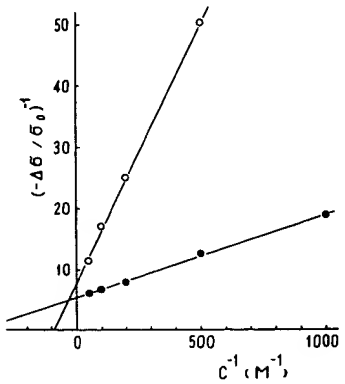

FIG. 4. Determination of the dissociation constants of maltose (filled circles) and sucrose (open circles) with concanavalin in a cross-linked film. $\Delta\sigma/\sigma_0$, relative changes of isometric stress; C, carbohydrate concentration. Other experimental conditions are presented in the footnotes to Table 1.

TABLE 1

Affinities of Protein and DNA Solid Samples for the Specific Ligands Compared with Affinities of Protein and DNA in Solution

Macromolecule	Ligand	Dissociation constants (mM)		Ref.
		In solids	In solution	
	Enzymes			
Hen egg-white lysozyme (crystal)[a]	GlcNAc	9.5; 109	15–70	(9)
	Glucose	440	2000	(10)
Pancreatic ribonuclease (crystal)[b]	2′(3′)-CMP	1.7	0.3	(11)
	Same in 0.1 *M* NaCl	3.3	2.0 (K_i)	(12)
Pancreatic carboxypeptidase A (crystal)[c]	Hipp-Phe-L	0.2; 1	0.05 (K_m)	(13)
	Phe	0.8; 2.5	2–5 (K_i)	(13,14)
Hg–papain (film)[d]	BAEE	20	14–17 (K_m)	(15)
	BAA	20	—	—
Acetylcholin esterase (film)[e]	Acetylcholine	1	0.14 (K_m)	(16)
Yeast hexokinase (film)[f]	Glucose in solutions A and B	0.2	0.2	(17)
	Glucose in solution C	1.1	0.2–6	(17)
Lactate dehydrogenase (film)[g]	NAD	10	0.4	(18)
	NADH	0.2	5×10^{-3}	(18)
Creatine kinase (film)[h]	Creatine	17	15.6	(19)
	CrP	20	8.6	(19)
	ATP	0.4	0.5 (K_m)	(19)
	Mg^{2+}	0.03	—	—
	Antibodies			
Monoclonal antibody (film)[i]	Theophylline	Less than 10^{-3}	10^{-5}	(20)
Monoclonal antibody (film)[j]	TNPS-Gly	Less than 10^{-3}	10^{-4}	—
	2,4-DNP	0.6	—	—
	2,5-DNP	No binding	—	—
	Transport proteins			
Parvalbumin (film)[k]	Ca^{2+}	10^{-4}; 1	10^{-6}–10^{-3}	(21)
	Mg^{2+}	10^{-1}	10^{-1}–10^{-2}	(21)
Avidin (film)[l]	Biotin	Less than 10^{-5}	10^{-12}	(22)
	Other proteins			
Human β_2-microglobulin (film)[m]	Ca^{2+}	4×10^{-2}	1×10^{-2}	(23)
	Cu^{2+}	5×10^{-2}	2×10^{-2}	(23)
Pea lectin (crystal)[n]	Glucose	2; 7	0.7–1.2	(24)
	Sucrose	3–4	—	
Concanavalin A (film)[o]	Maltose	2.7	0.35	(25)
	Sucrose	12	1.8	(25)
	Glucose	10	1.7	(25)
	Other macromolecules			
DNA from calf thymus[p]	Ethidium Br	10^{-3}	5×10^{-2}	(26)
	Actinomycin D	0.03	4×10^{-4}	(27)
	Acridine orange	0.3	0.02	(28)

Note. The comparison is at nearly identical values of pH and ionic strength; K_m and K_i, Michaelis or inhibition constants were taken for enzymes in solution.

[a] The data presented were obtained from elasticity measurements of triclinic and tetragonal crystals with a vibrating reed method (2,3). Testing of microtome cross sections of the triclinic crystal by the method described here revealed about a 5% increase in σ upon addition of a 0.1 M solution of GlcNAc in 0.05 M sodium acetate, 2% $NaNO_3$, pH 4.5.

[b] Monoclinic $P2_1$ crystals of bovine RNase (Serva) were grown according to the procedure of Wlodawer *et al.* (29) and cross-linked in a 0.5% solution of GA on mother liquid for a week. Binding of 2′(3′)-CMP in 10 mM imidazole buffer (pH 7.0) resulted in a 20% increase in σ measured along the [010] direction and a 50% decrease in σ in the [001] direction. Addition of 0.1 M NaCl decreased K_d as shown.

TABLE 1—*Continued*

[c] The crystals of bovine pancreatic carboxypeptidase A were grown from commercial (Serva and Reanal) products and cross-linked for 7 h in a 0.2% solution of GA as described by Quiocho and Richards (30). K_d was measured in 20 mM veronal buffer, pH 7.5, 0.2 M NaCl in the sample cut along an unidentified crystallographic direction. Hipp-Phe-L caused a 40% decrease in σ and a 16% decrease in the Young modulus, whereas phenylalanine increased σ by 10%.

[d] See Ref. (4) for more detailed information.

[e] Film was made from acetylcholinesterase of bovine erythrocytes (Sigma) with 12% (w/w) sucrose added to the protein solution before drying and cross-linking for 1 h. The maximum increase in σ due to acetylcholine addition reached 20%. Pretreatment with diisopropyl fluorophosphate (10^{-3} M for a few minutes) resulted in a complete loss of the sensitivity to acetylcholine.

[f] Hexokinase (Fluka) mixed with glucose (3:1 by weight) was dissolved in water, dried, and cross-linked for 1 h. Testing the film in 50 mM acetate buffer, pH 5.5 (noted in the table as solution C); in 50 mM Hepes, pH 8.0, with 0.4 M NaCl (solution A); and in 10 mM phosphate buffer, pH 7.4, with 0.1 M NaCl (solution B) has shown a similar reversible 2–10% increase in σ in response to glucose addition. In contrast, GlcNAc produced changes in σ of opposite sign.

[g] Enzyme from pig muscle (a gift of Dr. E. A. Saburova), cross-linked for 1 h. Testing was done in 0.1 M phosphate buffer, pH 7.0, 5 mM dithiothreitol. Both NAD and NADH produced a reversible 3–10% decrease in σ. No effects of lactate and pyruvate at 10 mM concentrations were detected.

[h] Protein from rabbit muscles (kindly given by Dr. E. P. Chetverikova), cross-linked for 2 h. Films were tested in 50 mM glycine buffer, pH 9.0. A reversible decrease in σ accompanied the binding of all the ligands presented, except Mg^{2+}, which increased σ by more than 100%.

[i] Monoclonal antibodies to theophylline were kindly supplied by Dr. R. G. Vasilov. Data presented in Fig. 6 were obtained on films with protected amino groups. Before the film was made, the antibody was treated with a 400-fold excess of 2,3-dimethylmaleic anhydride according to (31). Dried film was cross-linked for 3 h at 25°C in a 0.1% solution of GA in 20 mM phosphate buffer, pH 8.5, saturated with Na_2SO_4. After a wash with water, the film was stored for a day in 20 mM 1,6-hexanediamine at pH 6 to deblock amino groups and protect them from reaction with GA groups. Films were tested in 10 mM phosphate buffer, pH 7.0, with 0.1 M NaCl. The hapten-induced 10% decrease in σ was only partially reversible: response to small concentrations of the hapten ($<10^{-6}$ M) was not restored after 2 h of washing with the buffer.

[j] An antibody against TNPS-Gly (produced by Dr. G. B. Krapivinskii) was concentrated by ultrafiltration to about 20 mg/ml, dialyzed against cold water with pH adjusted to 8.2 with NH_4OH, dried, and cross-linked for 2 h. Film washed with water was then stored in 10 mM phosphate, pH 7.0, with 0.1 M NaCl at 4°C. Testing in the same buffer revealed a 70–50% decrease in σ on binding of TNPS-Gly. The pH dependence of the effect had a maximum σ drop at pH 7, with about half of this value at pH 5 and 9. 2,4-DNP-induced changes in σ are negative at pH > 5, with a maximum drop in σ at pH 7.0 and positive at pH < 5. The effect of 2,5-DNP was very small and its concentration dependence showed $K_d \Rightarrow \infty$.

[k] Carp and pike parvalbumins were kindly provided by Dr. L. P. Kalinichenko. Films were fixed for 2.5 h and stored in dry state before use. Testing was done in 15 mM imidazole buffer, pH 7.0, 0.093 M KCl. Solutions with a concentration of the free Ca ions less than 10^{-6} were made using 1 mM Ca–EDTA buffer according to (32). Higher concentrations were made by addition of $CaCl_2$ to the buffer, containing 1.6×10^{-6} M free Ca^{+2}. The 30-fold Ca^{2+}-induced changes in σ, presented in Fig. 3, reflect about a 4% shrinkage of the film in 1 mM Ca solution from that in the EDTA solution. An extensive increase in tension in the Ca solutions was also accompanied by a 5-fold increase in the film rigidity.

[l] Commercial (Sigma) avidin film was cross-linked for 1 h and then washed for 2 days in 20 mM 1,6-hexanediamine, pH 7.0. The minimal biotin concentration detectable by the σ decrease in 50 mM phosphate buffer, pH 7.0, with 0.1 M NaCl was about 10 nM. Decrease in σ on a complete saturation of binding sites reached 80%. No restoration of the tension and sensitivity to biotin was found after the film was washed in the buffer for a few hours.

[m] β_2-Microglobulin was purified by Professor D. Vučelič and Dr. J. Hranisavljević according to (33) from the urine of a patient suffering from Balkan nephropathy. Among more than 50 substances screened for their ability to bind β_2-microglobulin (sugars, amino and fatty acids, nucleotides, and nucleosides), only polyvalent cations, such as Ca^{2+}, La^{3+}, and Zn^{2+}, were found to strongly bind the protein and to increase σ by 10–200% (testing was done in 10 mM Hepes or imidazole buffers with 0.1 M NaCl, pH 7.0). High affinity of the β_2-microglobulin for these ions was then verified by dynamic dialysis in solution (23).

[n] Pea lectin crystals [$P2_12_12_1$, grown from 10–15% ethanol solution (34)] were kindly provided by Dr. R. R. Riskulov. Crystals were cross-linked with 1% GA in 10 mM acetate buffer, pH 5.6, 15% ethanol, for 24 h. They were washed and stored in the same buffer at 4°C. Dissociation constants presented in the table were obtained when crystalline samples were tested in 10 mM acetate buffer, pH 5.6, with 1 mM $CaCl_2$ and 1 mM $MnCl_2$ added. No pH dependence of the sucrose-induced 30–40% decrease in isometric tension was observed in the pH range 5.0–8.0, measured in 0.12 M NaCl solution buffered with 10 mM phosphate, acetate, and borate. It is worth noting that amorphous films show a similar decrease in σ; however, the chemomechanical effect in films is smaller and less stable than that in crystals.

[o] Concanavalin A was a Sigma product (type IV). Film was cross-linked for 1 h, stored in dry state at −15°C, and tested in 50 mM phosphate buffer, pH 6.8, 0.1 or 1 M NaCl, 0.1 mM $CaCl_2$, and 0.1 mM $MnCl_2$. As seen in Fig. 4, addition of sugars results in a 10–20% decrease in σ, which can be well represented as binding with a single site. Ionic strength seems to have no appreciable effect on sugar binding.

[p] DNA was kindly provided by Dr. V. I. Bruskov. As shown in Fig. 7, ligand-induced changes in the tension of DNA films cannot be described as adsorption isotherms, since binding of ethidium shows a reversal of σ changes at concentrations exceeding 10^{-5} M, whereas actinomycin and acridine orange binding are highly cooperative. Figures presented in the table indicate the smallest concentration producing a notable mechanical effect in DNA films tested in 20 mM phosphate buffer, 0.1 M NaCl, pH 7.0. Washing with buffer does not completely restore the initial tension and the sample remains colored. The effect of high concentrations of ethidium ($>10^{-5}$ M) is reversible. Decrease in tension at low concentrations of ethidium is completely restored only after the sample is boiled for a few minutes in 1–2 M NaCl solution. It completely decolorizes after such a procedure. Films cross-linked with GA vapor for a day and then washed with 20 mM glycine solution show the same effects of ethidium and acridine orange as uv-treated films.

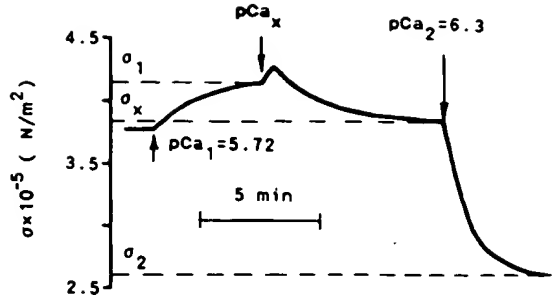

FIG. 5. An example of the measurement of the concentration of free Ca ions in a solution by comparing isometric stress (σ) in carp parvalbumin film in solutions with different concentrations of free Ca ions. pCa_1 and pCa_2 denote two EDTA buffers with known pCa; pCa_x denotes application of buffer with unknown pCa. Its concentration calculated by the interpolation formula from mechanical data is $pCa_x = 5.9$. Flame adsorption spectrometry (Perkin–Elmer instrument) revealed $pCa_x = 5.8$. Other experimental conditions are presented in the footnotes to Table 1.

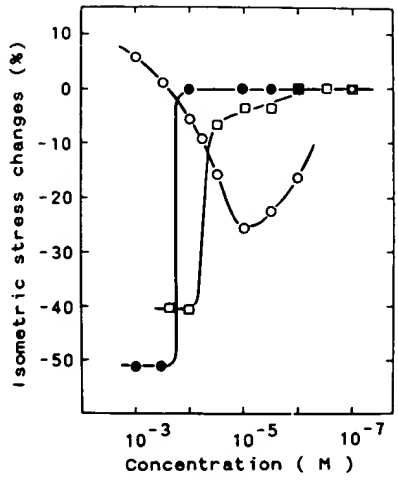

FIG. 7. Changes in isometric stress in calf thymus DNA film upon addition of different concentrations of intercalating compounds. Open circles, ethidium bromide; filled circles, acridine orange; squares, actinomycin D. Other experimental conditions are detailed in the footnotes to Table 1.

sal character. However, different mechanisms may underlie the effect in different samples. Binding-induced changes in the shape of protein molecules due to displacement of domain or subunit positions may be considered one mechanism of binding-induced deformation in protein solids. The effect of glucose on hexokinase films and that of phosphoglycerate on films of phosphoglycerate kinase may be explained by such a mechanism, according to the X-ray data (35,36). Changes in elastic properties of protein molecules as a result of ligand binding may be another possible mechanism of chemomechanical effects, as has been demonstrated for the binding of competitive inhibitor to lysozyme (2). Ligand-induced changes in intermolecular forces within solids may also result in deformation and/or elasticity changes of the samples. For example, binding of charged ligands to protein will give rise to a decrease or increase in the total charge of the macromolecules and thus to swelling or shrinking of the specimen. The larger effect of charged TNT-Gly than that of uncharged TNT may reflect a contribution of this mechanism. Mechanical effects of substrates in enzymatically active films (of acetylcholinesterase and carboxypeptidase) might include contribution of the reaction products. Obviously, a binding constant obtained with these films should be considered effective.

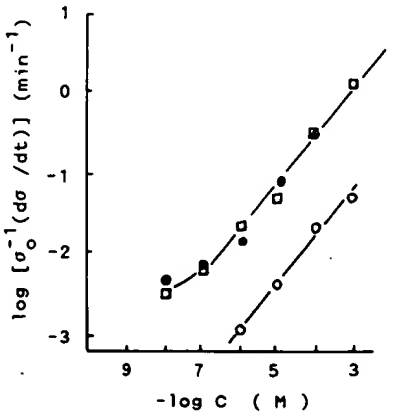

FIG. 6. Concentration dependence of the rate of relative isometric stress changes, σ_0^{-1} ($d\sigma/dt$), in protein films with a high affinity to specific ligands. Open circles, monoclonal antibody to theophylline in the hapten solutions; filled circles, monoclonal antibody to 2,4,6-trinitrophenyl-1-sulfonylglycine in the hapten solution; squares, avidin film in biotin solutions. Other experimental conditions are specified in the footnotes to Table 1.

Although the origin of the chemomechanical effect in each particular case is interesting from biochemical and biophysical perspectives, such a detailed analysis was beyond the scope of this paper and remains to be done. It is this diversity of mechanisms that makes the chemomechanical effect such a widespread phenomenon in protein and DNA solids and gels. With possible analytical applications of these effects in mind, we concentrate on a discussion of the practical problems of the specificity of the effects, their stability, interference from unspecific factors, and physical limitations on the minimal concentration of ligands detectable with the chemomechanical method.

Specificity. Although a special study of the specificity of ligand interactions with proteins and DNA in solid cross-linked samples is forthcoming, there is much evidence strongly supporting the high specificity of chemomechanical effects. First, we can see from Table 1 that in the majority of ligand–protein (DNA) pairs tested, the difference between binding constants obtained in mechanical measurements and those known from the

literature for interaction in solution does not exceed an order of magnitude, whereas binding constants themselves vary from 10^{-2} to 10^{-9} M. Second, in all cases in which more than one ligand was tested for interaction with a protein sample, there is a good correlation between the affinities of the ligands in solution and those in the solid sample. Thus, the lysozyme sample "distinguishes" GlcNAc and glucose, as does parvalbumin film with Ca and Mg ions, and the hexokinase film response is of a different sign for glucose and GlcNAc solutions. The interaction of concanavalin film with three saccharides, creatine kinase with creatine, and ATP shows the same hierarchy of affinities as that shown in solution. Even more refined characteristics of ligand–protein interaction are retained in solid samples. For example, the affinity of hexokinase for glucose decreases both in solution and within cross-linked films upon pH decrease. The same is true for the affinity of 2′(3′)-CMP for ribonuclease: upon addition of salt it decreases both in crystal and in solution (see Table 1). The most illustrative example of the stereospecifity of the chemomechanical effect is a comparison of the effects of TNT-Gly, 2,4-DNP, and 2,5-DNP in the film of monoclonal antibody to TNT-Gly. Whereas 2,4-DNP with two nitro groups arranged as in natural hapten, TNT-Gly, produces a mechanical effect in the film, its metaisomer, 2,5-DNP, is not able to produce marked specific mechanical effects.

Nevertheless, we must admit that there are some differences in the affinities of protein and DNA molecules measured in solutions and in solid samples. They may reflect changes in the properties of protein molecules during sample preparation (drying, cross-linking) as well as different conditions for binding within solid samples (due to steric hindrances, changes in the composition of intracrystalline solution due to the Donnan potential, etc.). The importance of diminishing the influence of these factors warrants a more detailed discussion of their action.

Drying procedure. Drying by itself is not very harmful to proteins, especially if it is rapid and proceeds at low temperature (37) or with some protective substance, like carbohydrates, added to the solution (38). Nevertheless, concentrating the macromolecules during drying may cause changes in the tertiary structure favoring the formation of dimers and higher olygomers with modified properties. Yeast hexokinase may present an example of such changes: dimer formation results in a several-fold increase of its K_d with glucose (17). Another result of drying may be the formation of very densely packed solids impenetrable by ligand molecules or presenting steric hindrance for binding itself and for binding-induced changes in the conformation of protein molecules. These steric and diffusion limitations in solid samples, however, may be readily reduced by decreasing the packing density of macromolecules by adding glycerin or carbohydrates to protein solutions before drying.

Chemical modification. Modification of α- and ϵ-amino groups occurs when macromolecules are cross-linked with GA. In principle, such a modification may result in steric blocking or in considerable deterioration of the binding sites with amino groups involved. We should note, however, that the presence of amino groups at a binding site does not exclude the possibility of obtaining good chemomechanical effects in cross-linked samples. Cross-linked crystals of RNase with three amino groups in the active center (39) retained their ability to bind the competitive inhibitor, 2′(3′)-CMP, in the absence of any protection of the binding site. The active sites may also be protected either by addition of a specific ligand to protein solutions during sample preparation or by reversible blocking of amino groups via acetylation before cross-linking (31). Reagents other than glutaraldehyde (carbodiimides, uv irradiation, etc.) may be also tried for cross-linking to make more native samples.

Donnan equilibria. Donnan equilibria may greatly change the internal pH and concentration of ions in densely packed solid samples at low ionic strength. Thus, with a protein concentration in tetragonal lysozyme crystal of 55 mM, the concentration of positive bound charges within the crystal at pH 4.5 reaches Z = 0.55 M. At a salt concentration of 0.01 M outside the crystal, the internal concentration of counterions is about 50 times greater and the internal pH is increased by 1.7 units. Provided the ligand is charged, its effective dissociation constant in such a solid will change according to the relation

$$K_{d,eff} = K_{d,0}\exp(z\psi/kT). \quad [5]$$

Here $\psi \cong 25 \ln(Z/C_{ext})$ (mV) is the Donnan potential, z is the charge of the ligand, $K_{d,0}$ is the dissociation constant in the absence of the Donnan potential, and $C_{ext} \ll Z$ is the external ion concentration. To avoid these effects in studies of charged ligands, one should work at a pH close to the pI of the protein or use concentrated salt buffer solutions.

Unspecific effects. Changes in pH and/or ionic strength, high concentrations of chemicals like glycerol and urea, changing interaction of protein molecules with water, or protein stability may also result in considerable changes in the mechanical properties of samples, comparable to or even more extensive than those caused by specific binding. Therefore, it is necessary to control these solution parameters and to keep them constant during measurements of specific effects.

Factors limiting the sensitivity, reversibility, and response time of the method. These are analyzed in the Appendix. The analysis shows that the response time is

dependent mainly on ligand concentration and its affinity. At ligand concentrations less than 0.1 μM the time needed to reach equilibrium binding exceeds 0.5 h, making the measurement inconvenient. High affinity (K_d < 10 nM) results in an irreversibility of mechanical response due to the extremely long time required to wash ligand out of the sample. The lowest concentration, C_{min}, that can be detected with the chemomechanical method depends on the K_d and on the signal-to-noise ratio of the device. In the device described here, $C_{min} \geq 0.01\ K_d$. Another restriction on C_{min} originates from diffusion limitations. Theoretical analysis and experiments with avidin films show that C_{min} cannot be lower than 10 nM if we want the signal to be obtained within 5–20 min. Lower concentrations can be measured at the cost of an increase in the time of measurement or after considerable improvement in the measuring device.

The minimum amount of ligand that can be detected in a drop of solution with no flow is 40 fmol, according to the estimates in the Appendix.

Storage and stability. It is particularly important that cross-linked protein samples retain their chemomechanical activity for a long time when stored under conditions similar to those used for lyophilized proteins and crystalline suspensions. Lysozyme, RNase, and carboxypeptidase cross-linked crystals did not show any decrease in chemomechanical response after 2–5 years of storage at 4°C in the solutions similar to their mother liquids. Parvalbumin film was active after storage for a year in a dry form at room temperature. The example of papain, which retained its ability to bind substrate with the same affinity after enzyme activity was blocked with $HgCl_2$ (4), suggests that chemomechanical-sensitive elements can be more stable than those in enzyme electrodes and other sensors based on enzymatic activity.

Possible applications of chemomechanical effects. As described under Results a single measuring device equipped with a number of replaceable chemosensitive elements can be used successfully for a qualitative and quantitative analysis of the binding of different substances with enzymes, antibodies, and other protein or DNA molecules. This combination of the device and chemosensitive elements can be considered a *universal chemosensor.* Compared to enzyme electrodes, it may be less sensitive to poisons, and it can be made for ligands that are not substrates of any enzyme. Compared with other chemosensors using antibodies, the chemomechanical sensor has the advantage in the analysis of small haptens, since their binding with antibodies is difficult to detect with known sensors.

When designed to connect to the output of a chromatograph, this device could be used as a *specific chromatographic detector,* allowing the recognition and automatic measurement of the concentration of any given substance or a class of substances (herbicides in environmental analysis, poisons and drugs in clinical analysis, etc.). Such a detector may work both in water solutions and in organic liquids, since proteins within solid samples have been recently shown to retain their ability to bind specific ligands (40) and to catalyze enzyme reactions (41) in organic media.

The biochemical properties of newly isolated biopolymers can also be tested with this method rapidly and with little consumption of the materials. Thus, the device allows the *rapid primary screening of substances that specifically bind biopolymer molecules.* Such screening may be effective in the search for new drugs targeting human, bacterial, or viral protein or DNA molecules, in primary checking of the biological activity of newly synthesized compounds, in biochemical studies of the allosteric control of enzymes by metabolites, etc. Checking complex mixtures of substances for the presence of active components is also possible. Attachment of the chemomechanical device to a chromatograph would allow rapid recognition and isolation of the active substances from complex mixtures. Compounds affecting protein or DNA sample in sufficiently low concentrations could then be studied with routine methods in solution. We have recently successfully applied the procedure to the search for substances specifically interacting with human β_2-microglobulin (23). It is interesting to note that in this screening strong interaction of this protein with Ca^{2+} and other polyvalent cations did not affect uv adsorption, as CD and NMR spectra of the protein. This made the detection of Ca-binding ability by these methods impossible. Only dynamic dialysis enabled the observation of the binding in solution. This is a good illustration of an advantage of the method in detecting binding when conventional methods fail. The simplicity with which chemosensitive elements are made, the low amounts of proteins or DNA, and the short time required for the analysis with the chemomechanical method are other advantages. Thus, less than 0.1 mg of desalted protein is enough to make more than 10 samples in 1.5–3 h using the procedure described under Materials and Methods. Since one sample contains only 0.1–0.2 μg of protein, the amount of protein used for sample preparation may be considerably reduced, if necessary. Each sample can be used to check many substances, provided their interaction with the protein is reversible. Testing one solution usually takes a few minutes; 1–2 h is needed to measure the binding constant.

CONCLUSIONS

Cross-linked crystals, amorphous films, and gels of proteins and other biological macromolecules may be considered new types of seminatural polymer materials (42). They differ from synthetic polymers mainly in the

chemical specificity inherited from parent biological molecules. There is no doubt that ligand-induced changes in such materials are not limited only to those mechanical responses described here, but must be determined by changes in other physical parameters.

APPENDIX

Factors Limiting the Sensitivity, Reversibility, and Response Time of the Method

Limits of the measurements of ligand-induced deformation. Mechanical methods enable the registration of changes in mechanical characteristics of macromolecules with high precision. Theoretically, precision in the determination of the sample length, L, is limited only by the amplitude of thermal fluctuations of the length,

$$\langle \Delta L^2 \rangle^{1/2} = (kTL/ES)^{1/2}, \qquad [A1]$$

where k is Boltzman's constant, T is temperature, E and S are Young's modulus and the cross-section area of the sample. For a 1-mm-long sample with a cross section of 5×20 μm and modulus $E = 0.2$ GPa (42), $\langle \Delta L^2 \rangle^{1/2}/L = 1.4 \times 10^{-8}$. Provided that the relative changes in molecular dimensions revealed in the length changes are of the same order, we may conclude that for molecules of dimensions of 40 Å, changes in average dimensions as small as 6×10^{-7} Å may be observable.

In real measurements, sensitivity is limited mainly by the signal-to-noise ratio of a measuring device. According to Eq. [4], ϵ_L is calculated as a product of initial static strain, ϵ_0, into a sum of relative changes of isometric tension, $\Delta F/F$, and Young's modulus of the sample, $-(\Delta E/E) = (\Delta\kappa/\kappa)$. With $\epsilon_0 \cong 1$–3% and about 1% precision in the measurements of F and κ, we obtain 2–6×10^{-4} as the limit of sensitivity to ligand-induced deformation of the sample, ϵ_L. This corresponds to changes in average dimensions of 40-Å-long protein molecules of 0.01 Å.

Lower limit of detectable ligand concentration. Three factors may impose a limit on the minimal concentration, C_{min}:signal-to-noise ratio of the instrument, diffusion, and amount of ligand molecules in a small volume. We consider them separately under conditions of equilibrium and nonequilibrium binding.

To detect ligand binding, the signal, ΔF, must exceed the noise level, F_n. If the maximum value of the signal (tension changes, accompanying complete saturation of all binding sites in the sample) is ΔF_{max}, then under equilibrium conditions the concentration dependence of the signal will follow the adsorption isotherm,

$$\Delta F = \Delta F_{max}[C/(K_d + C)], \qquad [A2]$$

where K_d is the dissociation constant. At concentration $C \ll K_d$ we have for C_{min}

$$C_{min} \geqslant (F_n/\Delta F_{max})K_d. \qquad [A3]$$

The noise-to-signal ratio in our measurements of ΔF varied between 0.1 and 0.01, depending on the stability of the device and the amplitude of mechanical changes of the sample accompanying binding. This means that under equilibrium conditions $C_{min} > (0.1$–$0.01)K_d$. In practice, this relation can be used only for samples with $K_d \geqslant 10^{-6}$ M, since external and internal diffusion greatly increase the time needed to reach equilibrium at low ligand concentrations.

To roughly estimate the limitations on C_{min} and the time of analysis, τ_1, due to external diffusion, let us consider a case of very high affinity, $K_d \to 0$ and $C \gg K_d$, when all ligand molecules reaching the surface of the sample are adsorbed. To obtain a signal distinguishable from noise we must saturate more than $(F_n/\Delta F_{max})$ of all the binding centers, $m = bhLC_p$ (C_p is the concentration of binding centers within the sample; b, h, and L are the width, thickness, and length of the sample). Assuming that the main diffusion of ligand occurred through an unstirred layer, δ, along the least dimension of the sample (i.e., its thickness) we can estimate the flow, J, from Fick's law for one-dimensional diffusion:

$$J = D \text{ grad } C \cong DC/\delta. \qquad [A4]$$

According to (43) the thickness of the unstirred layer can be calculated as

$$\delta = (\nu b/V)^{1/2}(D/\nu)^{1/3}, \qquad [A5]$$

with ν denoting the kinematic viscosity of the solution and V the flow rate of solution around the sample.

Now τ_1 can be estimated from the equation describing a material balance; i.e., ligand molecules transported with the flow via two lateral surfaces of the sample during time τ_1 must bind more than $(F_n/\Delta F_{max})$ of all binding centers in the sample:

$$2\tau_1 JbL \geqslant (F_n/\Delta F_{max})m. \qquad [A6]$$

Substituting J and m in this unequality, we obtain for C_{min} detectable in time interval τ_1

$$C_{min} \geqslant (h\delta C_p/2D\tau_1)(F_n/\Delta F_{max}). \qquad [A7]$$

Taking the real parameters, $V \cong 10^{-2}$ m/s, $D \cong 5 \times 10^{-10}$ m^2/s, $C_p = 30$ mM, $h = 5$ μm, $b = 50$ μm, $\nu = 1 \times 10^{-6}$ m^2/s, we conclude that C_{min} cannot be lower than 3×10^{-6} (F_n/F_{max}) M, for $\tau_1 = 5$ min. Thus, external diffusion limitations do not allow us to detect ligands with concentrations lower than 10^{-8} M, provided that the measurement takes about 5 min. It is clear that at $K_d \neq 0$ and $C < K_d$, τ_1 for the same C_{min} will be higher, since not all

diffused ligand molecules are bound under this condition and the concentration gradient is lower.

Internal diffusion in the sample may also be a restrictive factor able to considerably increase the response time of large ligands. It has been shown previously that the effective coefficient of the diffusion of dye molecules within protein films and crystals, D_e [calculated from the motion of boundary of colored zone in samples stored in a 1 mM solution of dye (44)], is far more dependent on the size of the molecules than that in solution, varying between $D_e = 1.5 \times 10^{-12}$ and 10^{-16} m^2/s for dyes in bovine serum albumin cross-linked films. Under conditions of strong binding, $C \gg K_d$, $K_d \to 0$, the depth of the ligand-saturated zone in the sample will increase according to the simple relation

$$x^2 = 2(C/C_p)D_p t = 2D_e t. \qquad [A8]$$

D_p means here the diffusion coefficient within the ligand-saturated zone. To obtain signal we must saturate a part of the sample, $2x = h\ (F_n/\Delta F_{max})$, which gives us

$$\tau_2 = (F_n/\Delta F_{max})^2(h^2 C_p/8CD_p). \qquad [A9]$$

For a 6-μm-thick sample, with $C_p = 30$ mM, a signal-to-noise ratio of 0.01, and external concentration of ligand $C = 10^{-8}$ M, we may calculate that signal will be obtained in 5 min only for those ligands that have D_p higher than 4×10^{-12} m^2/s. Data presented in (44) show that this limit is reached for dyes of 300 Da in monoclinic lysozyme crystals and 500–600 Da in amorphous BSA films. To avoid this limitation we must prepare less densely packed samples: we may expect that in gels of proteins and DNA internal diffusion will not restrict the binding of small protein molecules as ligands.

Reversibility. The reversibility of the chemomechanical effect is limited practically by the time needed to wash bound ligand from the sample, τ_3. It is mainly dependent on internal diffusion of the ligand molecules at $C \ll K_d$. In the presence of free binding centers, the effective diffusion coefficient, D'_e, is strongly reduced (44):

$$D'_e = D/[1 + (C_p/K_d)]. \qquad [A10]$$

With a concentration of the binding centers $C_p = 30$ mM we obtain a 10^6-fold reduction of the diffusion coefficient from its solution value of about $D = 5 \times 10^{-10}$ m^2/s to $D'_e = 1.7 \times 10^{-16}$ m^2/s for dissociation constant $K_d = 10$ nM. The characteristic time needed to wash out a 6-μm-thick sample under such conditions is about $\tau_3 = h^2/8D_e = 7$ h. This might explain the observed partial irreversibility of the hapten binding to the monoclonal antibodies (see footnotes to Table 1). Of course, the binding ability of the samples with this or higher affinity to the ligands could be restored by prolonged washing. But this could be extremely time consuming: provided avidin molecules retain their affinity to biotin ($K_d = 10^{-15}$ M) in the film, more than 8000 years would be required to wash biotin out of the avidin film.

Although admittedly crude, these estimates give a reasonable explanation for the scale of binding constants, $K_d \geq 10^{-6}$–10^{-7}, that one may measure with the mechanical method or with any method using protein films a few micrometers thick. The same is true for minimal concentrations, $C_{min} \geq 10^{-8}$ M, detectable within a few minutes in experiments with avidin films.

Restriction due to the final amount of ligand in a small volume. A sample of $5 \times 50 \times 500$ μm contains about 4 pmol of protein. Provided 1/100 of the molecules bind ligand to generate a signal exceeding the noise level, we need only 40 fmol of the ligand for the analysis, and the minimal concentration of the ligand in a droplet of 40 μl must exceed 10^{-9} M. Of course, in a flow chamber there is no such limit.

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Endocrine reviews (UNITED STATES) May 1990, 11 (2) p326-53, ISSN 0163-769X Journal Code: 8006258
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2) **Role of protein phosphorylation and inositol phospholipid turnover in rat parotid gland proliferation.**
Purushotham K R; Zelles T; Humphreys-Beher M G
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Molecular and cellular biochemistry (NETHERLANDS) Mar 27 1991, 102 (1) p19-33, ISSN 0300-8177 Journal Code: 0364456

3) ***Mechanical detection of interaction of small specific ligands with proteins and DNA in cross-linked samples.**
Morozov V N; Morozova TYa
Institute of Theoretical and Experimental Biophysics, Academy of Sciences of the USSR, Pushchino, Moscow Region.
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Role of protein phosphorylation and inositol phospholipid turnover in rat parotid gland proliferation

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Abstract

The involvement of protein phosphorylation in isoproterenol (ISO)-mediated proliferation in the rat parotid gland was investigated by labeling the cells with [^{32}P] orthophosphate. An increased (4–6 fold) incorporation of the radiolabel was noted in the total parotid gland homogenates of ISO-treated animals when compared to controls. Plasma membrane, nuclear membrane and cytoplasm were isolated, the proteins separated by SDS/PAGE and the phosphoproteins detected by autoradiography. Two phosphoproteins with apparent M_r of 45 and 170 kDa were identified in the cytoplasm while the 170 kDa phosphoprotein also appeared as part of plasma membrane. Transfer of these proteins to nitrocellulose followed by Western blot detection with an antiphosphotyrosine monoclonal antibody showed reactivity with the 170 kDa region of the plasma membrane and cytoplasm. Separate *in vitro* studies involving incubations of rat parotid slices with 0.2 mM ISO and [^{3}H] myo-inositol for 1 min induced inositol phosphate hydrolysis resulting in a significant increase in inositol-bis and -tris phosphate production. Inositol phosphate production can be blocked by pre-incubation with a mixed β-adrenergic receptor antagonist but not with physiological concentrations of α- or β_1-specific adrenergic receptor antagonists, indicating the ISO effects are mediated through the β_2-adrenergic receptors. The inclusion of calmodulin antagonists along with ISO prevented the expression of cell-surface galactosyltransferase and retarded gland hypertrophy and hyperplasia. These results suggest that ISO treatment leads to the phosphorylation of target proteins which may be involved in signal transduction pathways leading to cell proliferation.

Abbreviations: $InsP_1$, $InsP_2$, $InsP_3$ – inositol mono-, bis-, and tris-phosphates, UDP – Uridine diphosphate, PMSF – phenylmethylsulfonylfluoride, SDS – sodium dodecyl sulfate, TFP – Trifluoperazine, P-tyr – phosphotyrosine, Gal Tase – galactosyltransferase

Introduction

The modification of protein by addition or removal of one or more phosphate groups at specific amino acid residues occurs at every stage in the transduction of an extracellular signal into a nuclear event resulting in cell proliferation. The protein kinases which phosphorylate cellular proteins are regulated by a variety of substrates and cofactors. Substrates for protein kinases include growth factor receptors [1], α- and β-adrenergic receptors [2], enzymes [3], cytoskeletal [4] and nuclear [5] proteins. Protein phosphorylation stimulated by various protein kinases has been shown to be involved

in a number of cellular functions. In rat parotid glands, β-adrenergic stimulation leads to the formation of cAMP, activation of cAMP dependent protein kinases and phosphorylation of specific target proteins that are thought to be involved in exocytosis [6], cell proliferation and differentiation [7] and regulation of cellular calcium levels [8].

Cellular functions are regulated for the most part by two phosphorylation pathways, namely adenylate cyclase and the phosphoinositide transmembrane signalling system. Earlier investigations from this laboratory have suggested that the chronic administration of β-adrenergic agonist, isoproterenol (ISO), to rats results in hypertrophy and hyperplasia of the parotid gland which are mediated by an increase in the cell surface β1–4 galactosyltransferase [9–11]. The events that occur in the transition of a normal cell from stasis to proliferation are 1) an increase in the transcription and alteration of the enzyme galactosyltransferase activity levels from Golgi complex to plasma membrane and 2) the interaction of the enzyme with a cell surface glycoprotein which may be similar to that of a receptor-ligand interaction [11–13]. However, it is not clear how the signal produced by such an interaction reaches the nucleus to modulate the processes such as DNA replication and gene expression, or how galactosyltransferase is targeted to the plasma membrane. Therefore the present study examines the significance of protein phosphorylation and the regulatory role of the signalling pathway in response to ISO-mediated acinar cell proliferation. Although the activation of cAMP-dependent protein kinases and phosphorylation of the proteins participating in the secretory function of the salivary glands have been observed in response to agonists or cAMP analogues [6, 14], the role of phosphatidyl inositol acting as a second messenger in salivary gland proliferation has not been studied in great detail. In electroporation studies, Uno *et al.* [15] attributed a specific role for phosphatidyl inositol in yeast cell proliferation. The phosphatidyl inositol specific phospholipase C has been shown to regulate cell division in normal and neoplastic cells [16]. Recently it has been shown that in isolated rat parotid acinar cells, $InsP_3$ levels are increased following β-adrenergic stimulation by a mechanism involving cAMP and Ca^{2+} mobilization [17].

In addition to cyclic nucleotide and phospholipid regulated protein kinases, there are kinases which are regulated by binding of ligands to receptors with an intrinsic kinase activity. These are the tyrosine kinases which transfer the ATP gamma-phosphate group to a tyrosine residue of the target protein. It has been demonstrated that these growth factor activated tyrosine kinases phosphorylate specific DNA binding proteins, which effect the transcription of specific mRNAs [18]. These tyrosine kinases are also involved in control of cell proliferation best exemplified by a subclass of human neoplastic malignancies [19].

In the present paper we analyzed the late incorporation of radiolabeled phosphate in ISO treated parotid acinar cells to examine the possible pathways leading to cell proliferation. We observed, *in vivo,* the specific phosphorylation of cytoplasmic proteins corresponding to 45 and 170 kDa and a protein with M_r 170 kDa unique to the plasma membrane of ISO-treated rat parotid glands. Interestingly, the 170 kDa protein was also detected by using a monoclonal antibody to the phosphotyrosine antigen by Western blotting techniques. This result and the increase in $InsP_3$ levels in separate *in vitro* studies involving the incubation of isolated parotid gland slices with ISO provide evidence suggesting involvement of protein phosphorylation and specifically the tyrosine kinases in the enlargement of parotid glands.

Experimental procedures

Materials

Radioisotopes, myo [2-^{3}H] inositol-1-monophosphate (specific activity = 5 Ci/mmol), [^{32}P] orthophosphoric acid (specific activity = 20.8 Ci/mmol) and [^{14}C]-UDP-galactose (specific activity = 2 mCi/mmol) were purchased from New England Nuclear (Boston, MA). The Sprague-Dawley rats were purchased from the University of Florida breeding colony. Antibody to phosphotyrosine was obtained from Boehringer Mannheim (Indianapo-

lis, IN). *d, l*-Isoproterenol, Trifluoperazine diHCl and N-(6-Aminohexyl)-1-napthalene sulfonamide (W-5) hydrochloride were supplied by Sigma Chemical Co., St. Louis, Mo, USA. Ultrapure chemicals for electrophoresis were obtained from Bio-Rad, Richmond, Calif., USA. All other reagents were of reagent grade quality.

Protein phosphorylation in total parotid gland

Male Sprague-Dawley rats (175–200 g) were maintained in the laboratory for 1 week prior to experimentation. One group of animals received i.p. injections of 0.5 ml ISO (10 mg/ml), twice a day for three days (ISO-2). A second group of animals received a single dosage of ISO (ISO-1), at the same concentration, 1 hr before killing. A third group of animals receiving injections of 0.5 ml saline served as the control group. All animals had access to food and water *ad libitum* throughout the experimental period. On the fourth day each animal received an i.p. injection of 50 μl (50 μCi) of [^{32}P] labeled orthophosphate 1 hr prior to ISO or saline injection. This allowed for the uptake of label into the giand so as to maximize the incorporation of [^{32}P] into cellular material upon stimulation with β-agonist. Two hr later the animals were killed by exsanguination.

Membrane preparation

Parotid glands were removed following morphological identification. Total membrane fractions were prepared at 4° C by homogenization of parotid tissues in 10 mM Tris-HCl buffer, pH 8.0 with a Dounce apparatus. To avoid protein degradation, protein inhibitors (1% aprotinin and 0.5 mM PMSF) were added to the homogenate. A low speed centrifugation at 500 × g was then performed to remove the connective tissue as well as unlysed cells. The resulting slurry was then centrifuged at 100,000 × g for 1 hr to recover total membrane.

Plasma membranes were isolated by the method of Arvan and Castle [20] which is a protocol designed specifically for isolation of plasma membranes from rat parotid gland [9, 12]. The total membrane pellet was resuspended in 5 ml of 0.5 mM $MgCl_2$, 1 mM $NaHCO_3$ (pH 7.4) containing 0.7 mM EDTA. The membrane slurry was centrifuged at 12,500 × g for 15 min. The pellet was adjusted to 1.38 M sucrose (125 ml total volume), overlayed with 0.3 M sucrose and centrifuged for 2 hr in a Beckman L7–55 ultracentrifuge at 50,000 × g. Plasma membranes were removed from the interface, diluted to 0.35 M sucrose in 0.5 mM $MgCl_2$, and 1 mM $NaHCO_3$ (pH 7.4) containing 1.7 mM EDTA, and centrifuged in a Beckman L7–55 ultracentrifuge for 2 hr at 150,000 × g. The final membrane pellet was resuspended in 10 mM Tris-HCl buffer, pH 8.0. The purity of the membranes prepared in this manner is documented elsewhere [12]. Briefly, the fraction was shown to be enriched 10 fold over total membranes for γ-glutamyltranspeptidase, an enzyme that Arvan and Castle [20] found to be an appropriate marker for parotid acinar cell plasma membranes.

The isolation of nuclear membrane was performed according to the procedure of Blobel and Potter [21]. In brief, the parotid glands were homogenized in 5 ml of TKM buffer, pH 7.4 (5 mM Tris, 2.5 mM KCl, and 5 mM $MgCl_2$) containing 0.25 M sucrose. The homogenate was filtered through cheesecloth and brought to a concentration of 1.62 M sucrose, layered over 2.2 M sucrose at the bottom and centrifuged at 60,000 × g for 1 hr. The pellet was resuspended in 5 ml of TKM buffer pH 7.4, sonicated for 30 seconds with intermittent cooling and centrifuged at 100,000 × g for 2 hr. The resulting supernatant constitutes the nucleoplasm and the residue corresponding to nuclear membrane was resuspended in 10 mM Tris-HCl pH 8.0. The nuclear fractions thus prepared were found to have a higher ratio of DNA to protein as reflected by the absorbances at 260 nm/280 nm. Also, this fraction was enriched with thymidine kinase activity [21]. Small portions (20 μl) of the cytoplasm, nuclear membrane, nucleoplasm and plasma membrane were taken for measurement of radiolabel incorporation by liquid scintillation counting. Total protein in the samples was assayed by a modification of the Low-

ry Method with bovine serum albumin as the standard [22].

Polyacrylamide gel electrophoresis

Proteins from the parotid plasma membrane, nuclear membrane, and cytoplasm were subjected to electrophoresis in 10% polyacrylamide gels by the Tris-glycine system of Pugsley and Schnaitman [23]. Samples for gels were made up using a concentration of 1 mg of protein/ml of sample buffer. A 35 μg sample of protein per well was routinely used in gel electrophoresis. The gels were subsequently fixed according to the procedure of Fairbanks *et al.* [24] and dried for 1 hr. Autoradiography was performed by exposing the gel to Kodak XAR film for 5d at − 80° C.

Western blotting with phosphotyrosine antiserum

A Western blot analysis [25] of the parotid membranes and cytoplasm was performed to identify specific phosphorylation of tyrosine in the phosphorylated proteins. 35 μg of the protein from the sample were electrophoresed on a 10% SDS-polyacrylamide gel [23]. The proteins were electrophoretically transferred at 17 V for 12 h to nitrocellulose [25].

Following electrophoretic transfer, the proteins were blocked by soaking the nitrocellulose in 5% low fat milk for 30 min. The nitrocellulose paper was incubated for 24 hr with mouse anti-phosphotyrosine (Boehringer Mannheim Biochemicals) diluted 1 : 500 in PBS + 5% instant low fat milk. The paper was incubated for 1 hr with peroxidase conjugated antimouse IgG (Sigma Chemical) diluted 1 : 350. The incubation was done in a heat sealed plastic bag and the nitrocellulose paper was washed with three changes of PBS. The immunoreactive proteins were visualized by incubating the nitrocellulose paper in PBS containing 0.02% H_2O_2 and 4 mM 4-chloro-1-naphthol.

Calmodulin-affinity chromatography

The cell lysates prepared from the control and ISO treated rats were solubilized overnight at 4° C in a buffer containing 1% (wt/vol) Triton X-100, 250 mM NaCl, and 20 mM Tris (pH 7.4). After this, the suspensions were centrifuged at 20,000 × g for 30 min. The supernatant was adjusted to 2 mM Ca^{2+} with $CaCl_2$ and applied to a calmodulin-agarose column ALD (Gibco-BRL) which had been pre-equilibrated with calcium containing buffer (20 mM Tris pH 7.4, 1 mM $MgCl_2$, 1 mM Dithiothreitol, 2 mM $CaCl_2$, and 0.1% (wt/vol) Triton X-100) at a flow rate of 10 ml/hr. The column was then washed with seven to ten volumes of calcium containing buffer and then eluted with EGTA containing buffer [20 mM Tris, pH 7.4, 1 mM $MgCl_2$, 1 mM Dithiothreitol, 10 mM EGTA and 0.1% (wt/vol) Triton X-100]. The eluates were concentrated in an amicon filtration unit and stored at − 80° C until used for polyacrylamide gel electrophoresis to estimate protein purity. The calmodulin-binding proteins from control and ISO, purified by affinity chromatography, were electrophoresed as described above. The gels were fixed and subsequently stained with Coomassie Brilliant blue R-250.

Ca^{2+}/calmodulin kinase activity

The calmodulin kinase activity was assayed by the method of Yamauchi *et al.* [26]. The reaction mixture, in a total volume of 50 μl, contained 50 μM [γ-^{32}P] ATP (2–6 × 10^5 cpm), 8 mM Mg $(CH_3COO)_2$, 0.5 mM $CaCl_2$, 0.1 mM EGTA, 50 mM Hepes buffer pH 8.0, 10 units of calmodulin (Sigma), and 0.5 μg of protein purified by calmodulin affinity chromatography as described above from ISO-treated parotid glands. One unit of calmodulin activity is defined as stimulating the activity of 0.016 activated units of phosphodiesterase 3′;5′-cyclic nucleotide to 50% maximum activity at 30° C in the presence of 0.01 mM Ca^{2+}, pH 7.5. Control reactions were carried out in the absence of calmodulin. The contents were preincubated at 30° C for 2 min prior to addition of enzyme. Incubation was carried out at 30° C for 90 s. The reaction was terminated by

the addition of 10 μl of 0.4 M EDTA and the incorporation of [^{32}P] into protein was measured using glass fiber filters followed by scintillation counting.

Galactosyltransferase assay

The activity of β1–4 galactosyltransferase was measured as previously described by Humphreys-Beher *et al.* [27]. In brief, membrane fractions were resuspended in 10 mM Tris/HCl, pH 8.0 at a final protein concentration of 200 μg/ml. The assay mixture (total volume 50 μl) contained 0.1 M MES, pH 6.3, 25 mM $MnCl_2$, 0.5% triton X-100, 1 mM UDP (1-^{14}C)$^-$ galactose (2 m Ci/mM), 10 mM ovalbumin as acceptor and 0–0.5 mg of the membrane preparation. After incubation at 37° C for 1 hr, the reaction was terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid, and followed by the recovery of ^{14}C incorporated material on glass fiber filters [27]. Enzyme specific activity was expressed as nanomoles of galactose incorporated/hr/mg of membrane protein. The plasma membrane galactosyltransferase was measured in intact cells by incubation with ovalbumin as acceptor and UDP-[^{14}C]-galactose as described by Marchase *et al.* [12].

In vitro *galactosyltransferase assay in parotid acinar cells*

Parotid glands from the control rat were identified by gross morphology, following pentabarbital injection and death by exsanguination. Intact cells were freed from connective tissue and dissociated with collagenase and hyaluronidase as described by Oliver *et al.* [28]. Cells were washed into Ca^{2+}, Mg^{2+}-free Hanks solution containing 10 mM $MnCl_2$. They were resuspended in the same buffer and approximately 10^6 cells were added to each plate. Cells were treated with 50 and 100 μmoles of TFP or W-5 separately to see if the calmodulin antagonists had any effect on the *in vitro* galactosyltransferase activity of parotid cells. Separate plates were incubated with either 0.1 mM ISO alone or in combination with the antagonist for 4 hr at 37° C. Later, 200 μM UDP-[^{14}C]-galactose and ovalbumin (10 mg/ml) were added and the incubations were continued for 1 hr at 37° C with intermittent mixing of cells. The reactions were terminated by the addition of 1 ml cold 10% TCA and the enzyme activity was determined as described above. The level of galactosyltransferase activity due to cell leakage was determined by measuring lactate dehydrogenase activity in the culture media [12]. The level of enzyme measured in the media was not more than 5% of lactate dehydrogenase assayed by cells ruptured by sonic lysis.

Incorporation of thymidine into DNA

In vivo DNA synthesis was followed in rat parotid glands by monitoring the incorporation of [^{3}H] thymidine into 10% trichloroacetic-acid precipitable counts. In addition to ISO treatment, groups of rats were injected with either trifluoperazine alone (10 mg/ml; 0.5 ml, twice a day) or in combination with ISO (20 min prior to ISO injection). Animals receiving injections of saline served as control. Injections of the ISO, trifluoperazine or trifluoperazine/ISO were followed by the intraperitoneal administration of 100 μCi (100 μl) of [^{3}H] thymidine. The animals were killed 4 hr following this injection, and their glands removed and homogenized at 4° C. One hundred μl were removed for trichloroacetic acid precipitation on glass-fiber filters, followed by scintillation counting for [^{3}H]-thymidine incorporation. Part of the sample was removed for protein assay and the rest of the sample was utilized for total and plasma membrane preparation.

Tissue preparation and incubation of parotid slices

In vitro measurement of inositol phosphates was accomplished by the procedure of Downes *et al.* [29]. Briefly, the parotid glands from the control group of animals were removed and then trimmed free of connective tissue, fat and lymph nodes and chopped on a tissue sectioner at a thickness setting of 50 μm. Parotid slices were preincubated at 37° C in a shaking waterbath with three changes of Krebs

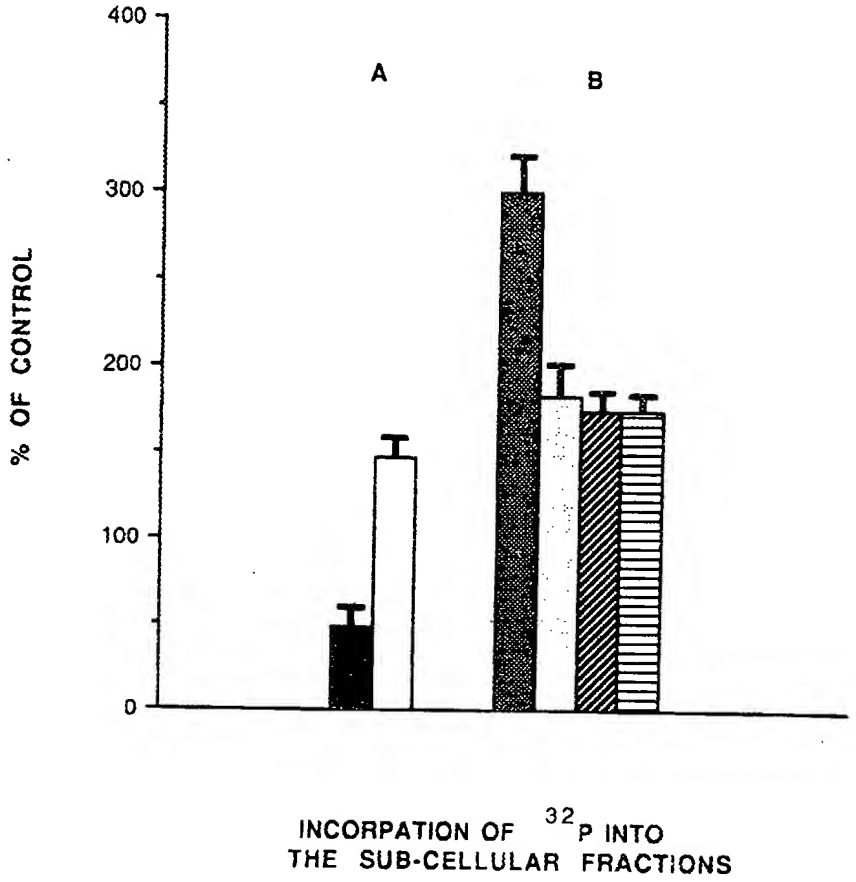

Fig. 1A. Histogram representation of [^{32}P] incorporation into rat parotid glands. After chronic isoproterenol administration (0.5 ml of 10 mg/ml i.p. injection, twice a day for 3 days) and 50 μCi of [^{32}P] orthophosphate injection for 2 hr, the rat parotid gland was isolated and homogenized in 10 mM Tris pH 8.0. The total membrane (100,000 × g pellet) was separated (▩) from supernatant (□).
Fig. 1B. Histogram representation of radiolabel incorporation into subcellular fractions. The cytoplasm (▩); plasma membrane (▨), nuclear membrane (▩) and nucleoplasm (▤) were isolated and the [^{32}P] incorporation into each fraction measured. Values presented as percentage of control, are mean ± S.D. of 3 experimental animals.

Hansleit Buffer (KHB) for 30 min. Five μCi of [^{3}H] myo-inositol was added to 25 μl of sample containing gently packed gland slices gassed with 95% O_2/5% CO_2. Two sets of tubes with the above contents were maintained with either 0.1 mM propranolol (mixed β_1- and β_2-antagonist) or 0.1 mM phenoxybenzamine (α-antagonist). Separate reactions, containing either of the above antagonists also included 0.2 mM ISO. A third set of reactions contained 0.2 mM ISO and the specific β_1-antagonist, atenolol, at a final 0.1 mM concentration. To a fourth set of tubes, 0.2 mM ISO alone was added and incubation of all sets of reactions continued at 37° C for 1 min. Incubations were terminated with 1 ml chloroform : methanol solution (1 : 2). Subsequent to this, 0.35 ml of chloroform and 0.35 ml of water containing HCl was added. 12.5 μg of phytate hydrolysate was added to get complete recovery of inositol phosphates. The aqueous and organic layers were mixed prior to centrifugation at 3000 rpm for 10 min using a Sorvall Instruments RC-3B centrifuge. From the upper aqueous phase of the above centrifugation, 0.75 ml was added to a 2 cm × 6 mm small column packed with AGX-8 Dowex, ion exchange resin (formate form). The inositol phosphates were separated by batch elution with increasing concentrations of ammonium formate/formic acid [29]. Each sample was solu-

bilized by the addition of 10 ml of counting cocktail 3a70B (Research Products International, IL, USA) and its radioactivity determined by scintillation counting. This method achieved excellent separation of $InsP_1$, $InsP_2$, and $InsP_3$ [30].

Results

Protein phosphorylation in parotid gland homogenates

In order to investigate protein phosphorylation that may be related to cell proliferation signals rather than exocytosis, animals were killed at longer time periods following radiolabeling than those routinely used following ISO treatment. The preliminary observations showed that the ISO treated animals killed 30 min following injection of [^{32}P] labeled orthophosphate demonstrated incorporation into the total homogenate of the parotid gland at twice the level of control animals. Radiolabel incorporation into the tissue homogenates 2 hr after injection showed a four fold increase compared to controls (8.3×10^6 cpm vs. 1.3×10^6 cpm respectively). To determine the level of incorporation into the total membranes compared with cytoplasm, the parotid gland homogenates were centrifuged at 100,000 × g. Higher incorporation of the label was found in the supernatant (cytoplasm, 10.2×10^5 cpm) than in the pellet (total membranes, 8.3×10^4 cpm) of homogenates of parotid glands isolated 2 hr after [^{32}P] injection (Fig. 1A). When the various subcellular fractions were analyzed for measurement of radioactivity, the greatest level of radiolabel incorporation was observed in the cytoplasm followed by plasma membrane and nucleus. (Fig. 1B). To explore further the relationship between ISO treatment and late protein phosphorylation, a series of experiments was performed varying the time of β-agonist injection under conditions of chronic administration. In the ISO-1 group, a decrease in the incorporation of radiolabel was noticed in the cytoplasm and total membranes similar to gland homogenates isolated from ISO-2 rats (data not shown).

As shown in Fig. 2, two proteins were labeled by [^{32}P] orthophosphate in the cytoplasm of ISO-treated rats corresponding to 45 and 170 kDa. In the case of control gland lysates, only one protein with M_r 45 kDa appears to be prominently labeled. It is apparent that the band at 45 kDa is more prominent in the ISO-2 sample, but the 170 kDa band is more prominent in ISO-1 than ISO-2 parotid glands. (Fig. 2A). This was consistent with the difference in incorporation of the label into the total membranes and also into the individual cellular fractions. The autoradiogram of plasma membrane preparation showed a distinct band at ~ 170 kDa in the ISO treated groups. (Fig. 2B). The nuclear membrane from ISO-2 treated animals showed phosphorylation of two distinct proteins of 140 and 170 kDa. (Fig. 2C).

To determine if the phosphorylation was specific for serine, threonine or tyrosine, detection of P-tyr moieties was performed with a monoclonal antibody to this unique antigen. Western blotting technique and detection with an antiphosphotyrosine monoclonal antibody showed distinct reactivity with the 170 kDa in both plasma membrane and cytoplasm (Fig. 3) but no reactivity with the nuclear fraction. This would suggest the nuclear proteins to be phosphorylated on either ser or thr residues as well as the cytoplasmic protein of 45 kDa. The presence of the 170 kDa protein in the cytoplasm of ISO treated animals could be due to the presence of an endosome fraction containing the internalized plasma membrane 170 kDa protein. The cytoplasmic fraction did not show additional activity of plasma membrane marker-enzymes over that observed in control cytoplasmic material.

When the total membranes of the parotid gland homogenates from control and ISO-treated rats were separated by polyacrylamide gel electrophoresis and autoradiographed, there was also a dark smear at the bottom (data not shown). This did not represent the free phosphate (which runs ahead of the dye front) but represented the incorporation of label into phospholipids. There was no detection of additional phosphoproteins, most likely due to the length of time between injection of β-agonist and the removal of the parotid gland from the rats.

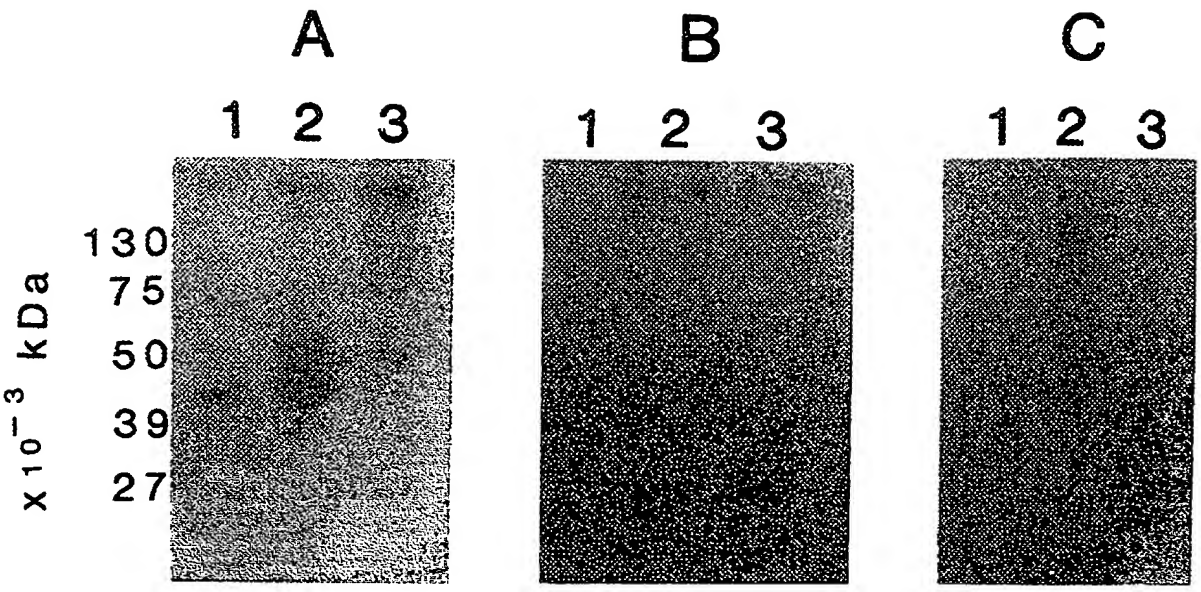

Fig. 2. Protein phosphorylation patterns in rat parotid glands stimulated by isoproterenol. Rats were injected with (1) phosphate-buffered saline, (2) chronic (ISO-2) or (3) a single (ISO-1) administration of isoproterenol as described in Experimental Methods Section. Proteins from subcellular fractionation were separated by polyacrylamide gel electrophoresis. The gels were dried and exposed to Kodak X-AR X-ray film at − 80° C for 5 days. The autoradiogram of cytoplasm (Fig. A), plasma membrane (Fig. B) and nuclear membrane (Fig. C) prepared from the above homogenates is presented. Thirty-five μg of protein was added to each well. Prestained molecular weight standards are: phosphorylase B, 130,000 Da; bovine serum albumin, 70,000 Da; ovalbumin 50,000 Da; carbonic anhydrase 39,000 Da; soybean trypsin inhibitor, 27,000 Da.

Turnover of phosphatidyl inositol

To confirm further the presence of labeled phospholipid, separate *in vitro* studies were conducted to determine the turnover of inositol phospholipids by incubating parotid slices from the control group of animals in the presence of the β-adrenergic receptor agonist ISO (Fig. 4). Incubation of parotid slices with 0.2 mM ISO induced phosphatidyl inositol hydrolysis, which resulted in a significant ($P < 0.001$) elevation in the levels of $InsP_2$ and $InsP_3$ as compared to the gland slices incubated without ISO in the medium.

To examine whether $InsP_3$ production was mediated through β-adrenergic receptors, we included the α-(phenoxybenzamine) and mixed β-(propranolol) adrenergic antagonists separately in the incubation in addition to ISO. Tubes with either of the antagonists alone were also incubated with the parotid slices, to compare the effects of the ISO combination. The addition of β-antagonist and ISO to the slices dramatically reduced ($P < 0.05$) the formation of $InsP_1$, $InsP_2$ and $InsP_3$. In parallel experiments, inclusion of the α-antagonist did not block the inositol phosphate levels as observed for the propranolol (Fig. 4). The inclusion of the specific β-adrenergic antagonist, atenolol (0.1 mM), had no effect on the ISO-induced inositol phosphate hydrolysis (data not shown), suggesting the response was mediated through a β_2-adrenergic receptor response.

Calmodulin antagonists

Since inositol phospholipid metabolism results in the mobilization of Ca^{2+} [17], which subsequently interacts with Ca^{2+}/calmodulin dependent protein kinases, it was of interest to determine what role these kinases might have on acinar cell proliferation. In *in vivo* experiments, injection of ISO caused a typical increase in cell surface galacto-

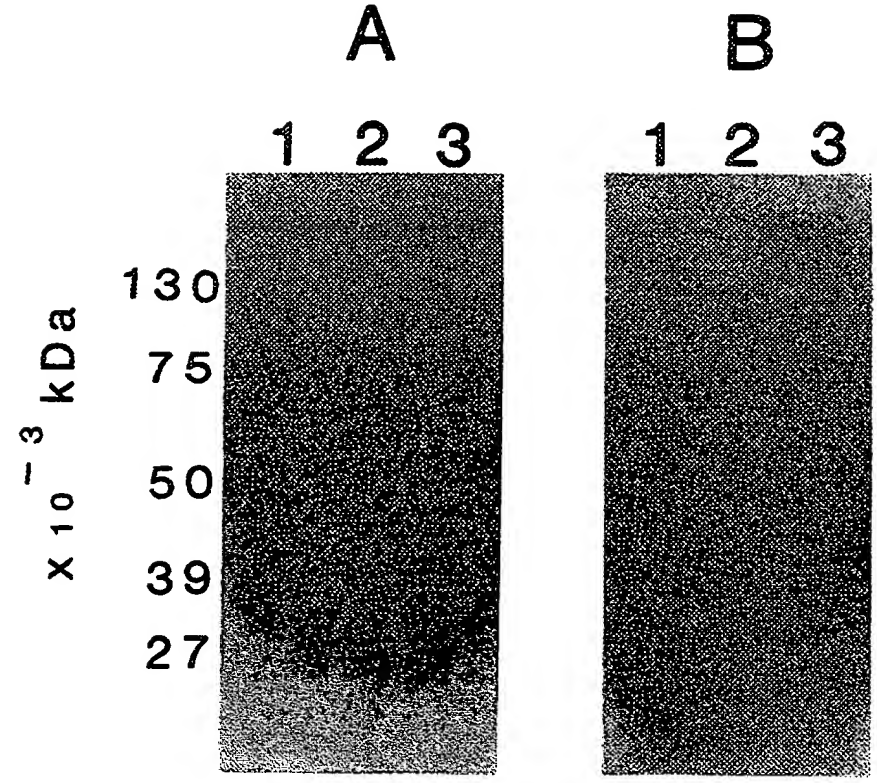

Fig. 3. Western blot detection of phosphotyrosine-containing proteins from parotid acinar cell lysates. 35 μg of protein from cytoplasm (Fig. A; 1 = control; 2 = ISO-2; 3 = ISO-1) and plasma membrane (Fig. B; 1 = control; 2 = ISO-2; 3 = ISO-1) separated by electrophoresis was transferred to nitrocellulose at 17 V for 12 hr before reacting with antibody to phosphotyrosine. Prestained molecular weight standards are the same as in Fig. 2.

syltransferase, gland hypertrophy and hyperplasia (Table 1). The incorporation of [^{3}H]-thymidine was used as a measure of DNA synthesis and thus an indirect assay of cell hyperplasia. The injection of the Ca^{2+}/calmodulin inhibitor, TFP, had no effect on basal levels of enzyme activity or acinar cell hypertrophy and hyperplasia. However when administered in combination with ISO, it effectively inhibited increases in gland weights, increased [^{3}H]-thymidine incorporation, and increased galactosyltransferase activity ($P < 0.05$) assayed from isolated total and plasma membrane fractions (Table 1). When cell lysates from control and ISO-treated animals were incubated with [^{32}P]-γ-ATP and an exogenous source of bovine galactosyltransferase, the enzyme was specifically labeled by ISO-treated but not control lysates (data not shown).

To show the effects of the inhibitor were specific for parotid acinar cells and not an *in vivo* metabolite of the drug, intact isolated parotid cells were treated *in vitro*. Incubation with 100 μmoles of ISO showed a 2 fold increase in plasma membrane β1–4 galactosyltransferase activity when compared to control. With either of two calmodulin antagonists TFP or W-5 (100 μmoles) alone in the culture media, enzyme activity was slightly lower than control values. However, when added together with ISO, they dramatically reduced the enhanced levels of galactosyltransferase activity ($P < 0.05$) compared to the effect of ISO alone. Both the antagonists exhibited a dose response pattern in inhibition (Fig. 5). These results suggest a role for increased $InsP_3$ and subsequent Ca^{2+} mobilization in regulating the targeting of galactosyltransferase from the Golgi complex to plasma membrane and mediation of cell proliferation.

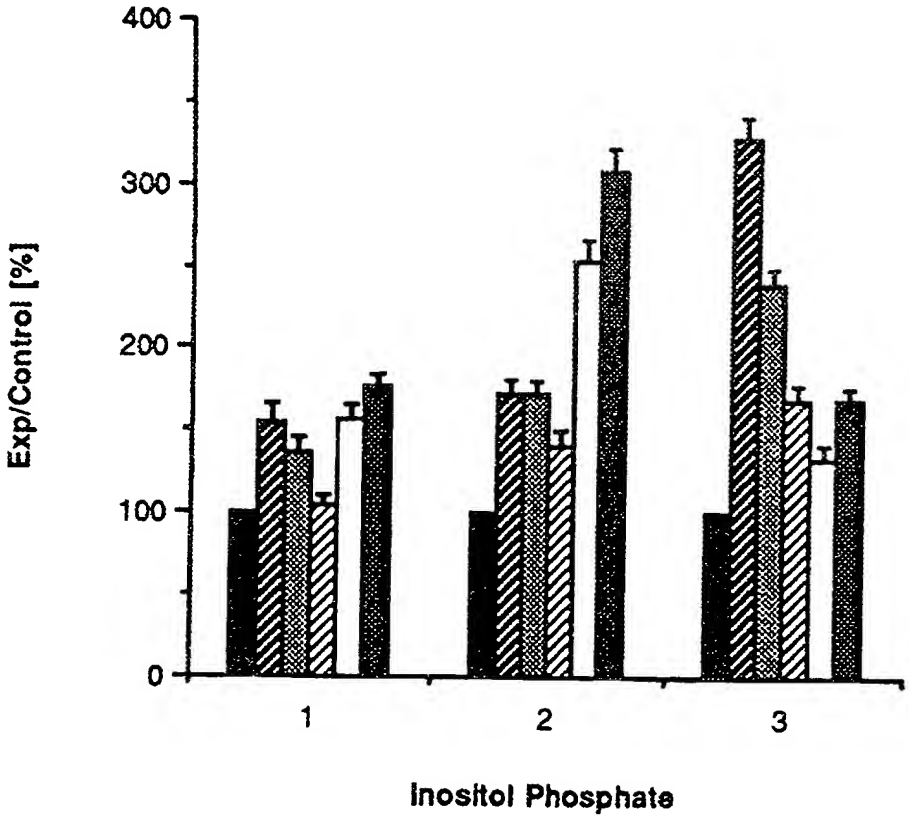

Fig. 4. Radiolabeling of parotid gland slices in the presence of adrenergic antagonists with simultaneous ISO stimulated incorporation of [^{3}H] inositol into phosphoinositides. Slices were labeled with [^{3}H] inositol for measuring radioactivity into inositol phosphates as described under Experimental Methods. Samples were incubated at 37° C for 30 min and exposed for 1 min to buffer (control ■) or various agonist and antagonist combinations; 0.2 mM ISO (□); 0.1 mM propranolol (□); 0.1 mM propranolol and 0.2 mM ISO (□); 0.1 mM phenoxybenzamine (□); 0.1 mM phenoxybenzamine and 0.2 mM ISO (■). Values of $InsP_1$, (1) $InsP_2$ (2) and $InsP_3$ (3) are expressed relative to [^{3}H] inositol present and are shown relative to the control values. Control values (mean ± S.D.) in these experiments are 56 ± 11 ($InsP_1$), 393 ± 56 ($InsP_2$), 693 ± 190 ($InsP_3$) dpm. Values represent the results of 3 separate experimental determinations.

Table 1. Effect of isoproterenol and the calmodulin inhibitor TFP on certain parotid gland parameters

Gland function	Control	TFP	ISO	TFP/ISO
Parotid gland weight (g)	0.25 ± 0.03	0.25 ± 0.01	0.88 ± 0.05[2]	0.41 ± 0.03[3]
^{3}H-thymidine incorporation (cpm)/gram parotid gland	18,585 ± 325	18,895 ± 1556	73,030 ± 410[2]	39,640 ± 1350[3]
β1–4 galactosyltransferase total membrane nmoles/hr/mg protein	0.17 ± 0.01	0.17 ± 0.01	0.4 ± 0.03[2]	0.16 ± 0.04[3]
β1–4 galactosyltransferase plasma membrane[1] nmoles/hr/mg protein	0.09 ± 0.01	0.10 ± 0.01	1.96 ± 0.09[2]	0.12 ± 0.02[3]

[1] Plasma membrane fractions were assayed for the level of sialyltransferase to determine contamination from Golgi membranes [9]. Each value is a mean ± SD of 3 animals.
[2] $P < 0.05$ between control and ISO;
[3] $P < 0.05$ between ISO and TFP/ISO. TFP values were not significantly different from control.

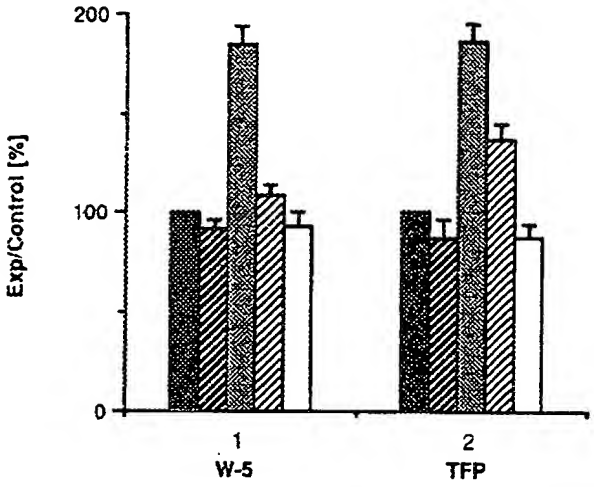

Fig. 5. Effect of TFP and W-5 on the ISO-stimulated galactosyltransferase activity in intact isolated parotid cells. Parotid cells were isolated free of connective tissue and were incubated at 37° C with only media control (■) 0.1 mM TFP or 0.1 mM W-5 (□); 0.1 mM ISO (□); 0.05 mM TFP or 0.05 mM W-5 + 0.1 mM ISO (□); 0.1 mM TFP or 0.1 mM W-5 + 0.1 mM ISO (□). After 4 hr [^{14}C] galactose and ovalbumin were added to intact cells and incubations were continued for 1 hr. The reaction was stopped with ice-cold 10% TCA and the radioactivity of the aliquots measured on glass fiber filters. Values (means ± S.D. of 3 animals) are expressed as percent of control (the specific activity of control is 0.18 ± 0.03 nmoles/hr/mg of protein.)

Identification of a unique calmodulin binding protein following ISO treatment

In an attempt to identify Ca^{2+}/calmodulin binding proteins following ISO treatment, proteins were again separated by SDS polyacrylamide gels and transferred to nitrocellulose. Using a biotinylated calmodulin, several proteins were detected in samples from control and ISO-treated parotid glands (data not shown). When the biotinylated calmodulin was incubated in the presence of the inhibitor TFP, binding to a 75 kDa protein from ISO-treated animals was uniquely prevented. The inclusion of EGTA blocked the binding of the calmodulin to all proteins as expected.

Chromatography on a calmodulin-agarose affinity matrix purified the 75 kDa protein from ISO-treated cells (Fig. 6). The chromatography of the control cell lysates over this column did not result in the detection or purification of this particular protein. The addition of [^{32}P]-γ-ATP, 0.5 mM $CaCl_2$, 10 U of calmodulin (Sigma) and bovine galactosyltransferase resulted in the specific phosphorylation of the added transferase substrate (Table 2). A second protein, histone was also a substrate of the enzyme but not to the extent exhibited by bovine galactosyltransferase. As a control, BSA was included in the reaction with no apparent affinity for the enzyme. The kinase activity was dependent on the interaction of Ca^{2+} and calmodulin as well as reflecting the ability of TFP to inhibit the reaction.

Discussion

Our results show an enhanced incorporation of the [^{32}P] label into parotid giand subcellular fractions as late as 2 hr following β-adrenergic agonist treatment in the rat. Greater incorporation of the radiolabel in ISO stimulated salivary glands of rabbits, rats and guinea pigs under *in vivo* and *in vitro* conditions has been reported by others at times up to 15 min following agonist treatment [14, 31–33]. The incorporated phosphate is utilized for the phosphorylation of target proteins to carry out specific cellular functions. We identified here the phosphorylation of proteins with molecular weights 45 and 170 kDa in the plasma membrane and cytoplasm that may be involved in the parotid gland acinar cell proliferation. The labeled ~45 kDa protein may be an isozyme of protein kinase A in rat salivary gland previously observed by Quissell *et al.* [34] in acinar cell phosphorylation experiments.

Dowd *et al.* [14] observed, in isolated rabbit parotid microsomes, the phosphorylation of 2 distinct proteins with M_r 34 and 30 kDa in the presence of ISO. However, these low molecular weight proteins were not detected by us in rat parotid giands. The reason for this may be that phosphorylation of the rat parotid gland plasma membrane and cytoplasmic proteins is more readily detected under *in vitro* conditions enriching specifically for the microsomal protein as compared to *in vivo*. *In vivo* incorporation will also result in a greater dilution of radiolabel than *in vitro* experiments with dispersed cells. Phosphate incorporation was measured in

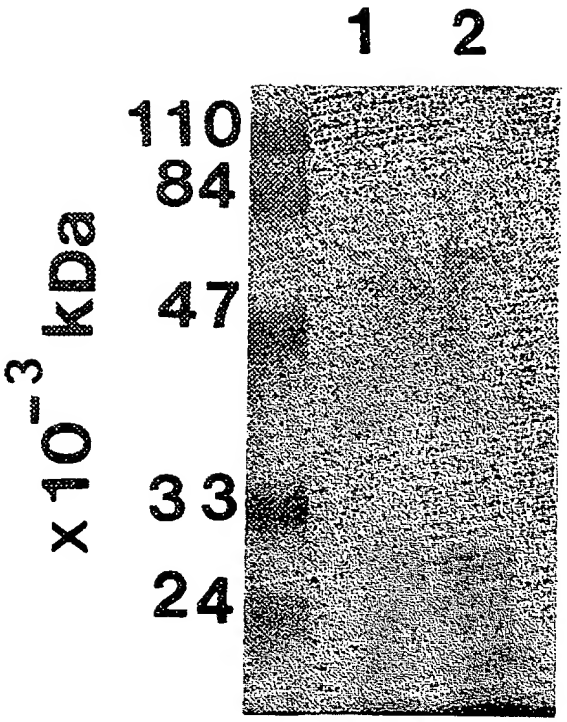

Fig. 6. Identification of a purified 75 kDa protein kinase in the ISO treated rat (ISO-2) parotid gland following electrophoresis on 10% SDS-polyacrylamide gel and stained with Coomassie blue R-250. Cell lysates from control (1) and ISO (2) treated rats were solubilized with 1% Triton X-100 and applied to calmodulin-agarose column. The eluate was concentrated and 2 μg of purified protein was added to the gel. Prestained molecular weight standards are: phosphorylase B, 110,000 Da; bovine serum albumin, 84,000 Da; ovalbumin, 33,000 Da; carbonic anhydrase, 24,000 Da.

our experiments 1 or 2 hr after ISO stimulation as compared with 10 min or 30 min, by Dowd *et al.* [14]. Additionally, there may be a species difference in parotid gland labeling between rat and rabbit.

Soling *et al.* [33] showed that β-adrenergic stimulation of guinea pig acinar cells activates protein kinases as a result of specific phosphorylation events. The membrane bound cAMP dependent protein kinase A (PKA) is known to be activated by β-adrenergic receptor stimulation [34]. From these reports it may be concluded that proteins are phosphorylated in response to β-adrenergic receptors by a mechanism involving cAMP dependent PKA. The subsequent Ca^{2+} mobilization has been shown to be involved in secretory vesicle mobilization and fusion to the plasma membrane in response to gland stimulation.

More recently the phosphorylation catalyzed by protein kinase C (PKC) of phosphoinositides, upon activation of cAMP dependent PKA, has been shown to regulate various cellular processes such as exocytosis, cellular proliferation and differentiation [7]. Berridge and Taylor [35] outlined a general pathway of receptor activation when an external stimulus activates a receptor. Stimulation of phosphoinositidase C (PIC) through a GTP binding protein causes the PIC to hydrolyse $InsP_2$ to $InsP_3$ and diacylglycerol. This is the first transduction event at the plasma membrane where the signal is translated into intracellular second messengers. The second translation event concerns the way in which the second messengers mediate their effects. In the case of $InsP_3$, it is mediated by Ca^{2+}. Calcium enters cytosol via endoplasmic reticulum near the plasma membrane, and the release of Ca^{2+} is mediated by $InsP_3$ acting through specific receptors located in the endoplasmic reticulum. Our results showed a significant increase in the levels of $InsP_3$ and $InsP_2$ when isolated rat parotid slices were stimulated with 0.2 mM ISO, suggesting the β-adrenergic agonists follow the above general pathway. Evidence in support of this comes from the studies of Berridge [36] and others [7, 17, 33, 37],

Table 2. Incorporation of [^{32}P]-γ-ATP into protein substrates by purified 75 kDa protein kinase

Condition[1]	cpm/μg substrate[2]
no Ca^{2+}/bovine Gal Tase	27,000
0.2 mM Ca^{2+}/bovine Gal Tase	44,000
0.5 mM Ca^{2+}/bovine Gal Tase	67,000
0.5 mM Ca^{2+}/bovine Gal Tase/no calmodulin	19,000
0.5 mM Ca^{2+}/bovine Gal Tase/TFP[4]	13,000
0.5 mM Ca^{2+}/Histone	39,000
0.5 mM Ca^{2+}/BSA[3]	5,000

[1] Incubation conditions included 10 units of calmodulin.
[2] All values expressed as mean of two experimental determinations and have been corrected for background counts associated with the addition of no substrate protein. Values were rounded to nearest thousand.
[3] BSA, bovine serum albumin.
[4] TFP concentration used was 0.025 mM.

who reported increased levels of $InsP_3$, and mobilization of intracellular Ca^{2+} following β-adrenergic stimulation. Phospholipase C activation and subsequent inositol phosphate hydrolysis accompanied by Ca^{2+} mobilization can also occur through plasma membrane receptor tyrosine kinases [38]. One interesting observation from our studies would be the finding of tyrosine phosphorylation of plasma membrane proteins.

Recently Adams *et al.* [39] have shown the galactosyltransferase activator cDNA [40] to have extensive homology to calcium/camodulin dependent protein kinase which affects the expression of β1–4 galactosyltransferase [41]. We reported earlier the constitutive expression of cell surface β1–4 galactosyltransferase and increased expression of the galactosyltransferase activator to be involved in the proliferation of parotid gland under a chronic ISO drug regimen [13, 40]. When we injected the animals with a calmodulin antagonist, TFP, along with ISO, the parotid gland weight, galactosyltransferase in the plasma membrane and the [^{3}H]-thymidine incorporation were all reduced when compared with the ISO treatment alone. The increased $InsP_3$ and the indirect evidence for the release of Ca^{2+} (and subsequent interaction with calmodulin) through the above studies suggest that the signal for proliferation involving surface galactosyltransferase as well as protein exocytosis may in part be mediated through an inositol phosphate cascade. The detection of a 75 kDa calmodulin binding protein with kinase activity using galactosyltransferase as a substrate suggests a possible mechanism for the regulation of cell surface enzyme during active cell division. The molecular weight of the kinase purified in this study is consistent with the reported molecular weight for the human recombinant GTA protein [41].

From these observations, we propose the following model for cell proliferation when the rats are treated with chronic ISO. Acinar cell proliferation may occur by the *de novo* expression of the galactosyltransferase activator kinase. Initiation of gene expression would be predicted to occur through β-adrenergic receptor mediated increases in cAMP. cAMP would trigger changes in transcription through the binding to a CRE element at the 5′ end of the GTA chromosomal sequences, similar to those found in the regulation of proline-rich protein expression [42]. Subsequent alterations in subcellular localization of galactosyltransferase to the plasma membrane may be mediated by Ca^{2+} mobilization from intracellular inositol phosphate turnover and interaction with calmodulin and the GTA kinase. The signal for cellular proliferation mediated by increased expression of cell surface galactosyltransferase may then be potentiated by the tyrosine kinase activity of a growth factor receptor. We are currently testing this hypothesis.

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1) **Signal transduction mechanisms of Ca2+ mobilizing hormones: the case of gonadotropin-releasing hormone.**
Naor Z
Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel.
Endocrine reviews (UNITED STATES) May 1990, 11 (2) p326-53, ISSN 0163-769X Journal Code: 8006258
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Document type: Journal Article; Review; Review, Tutorial

2) **Role of protein phosphorylation and inositol phospholipid turnover in rat parotid gland proliferation.**
Purushotham K R; Zelles T; Humphreys-Beher M G
Department of Oral Biology, University of Florida, Gainesville 32610.
Molecular and cellular biochemistry (NETHERLANDS) Mar 27 1991, 102 (1) p19-33, ISSN 0300-8177 Journal Code: 0364456

3)*** **Mechanical detection of interaction of small specific ligands with proteins and DNA in cross-linked samples.**
Morozov V N; Morozova TYa
Institute of Theoretical and Experimental Biophysics, Academy of Sciences of the USSR, Pushchino, Moscow Region.
Analytical biochemistry (UNITED STATES) Feb 14 1992, 201 (1) p68-79, ISSN 0003-2697 Journal Code: 0370535

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Signal Transduction Mechanisms of Ca^{2+} Mobilizing Hormones: The Case of Gonadotropin-Releasing Hormone*

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Introduction

Hormones, neurotransmitters, growth factors, antigens, and light exert their diverse biological effects by producing "second messenger" molecules which amplify and propagate the receptor signal into the cell biochemical network. The "second messengers" [*e.g.* cAMP, cGMP, Ca^{2+}, and diacylglycerol (DG)] activate respective protein kinases which are the key enzymes in the regulatory network of the living organism (1). Protein kinases (~100 have been described) are divided into serine/threonine and tyrosine-specific kinases (2). The main members of the first family are activated by second messengers, whereas several members of the tyrosine-specific kinases are associated with growth factor receptors or are encoded by retroviral oncogenes or the cellular homolog counterparts. The kinases catalyze the transfer of phosphate from ATP to serine, threonine, or tyrosine in the substrate proteins, and phosphatases remove the phosphate group (3). The phosphorylation-dephosphorylation cycle enables the cell to alternate from resting to activated state (or vice versa) according to the stimulus input.

In this review we will present general considerations concerning Ca^{2+}-mobilizing hormones, and specific examples will be presented from the area of GnRH action. GnRH is the first key hormone in the reproductive system; nevertheless, its mechanism of action is not known. The complex interaction of neurotransmitters, steroid hormones, and neuropeptides involved in the pulsatile nature of GnRH release from the median eminence is well recognized (4, 5). The precursor protein for GnRH was identified by recombinant DNA techniques and was found to contain a signal peptide, the GnRH decapeptide, and a GnRH-associated peptide (GAP) thought to be involved in inhibition of PRL secretion (6–8).

Several reviews have promoted the idea that extracellular Ca^{2+} is necessary and sufficient for mediating the exocytotic response of the neurohormone (9–11). However, recent evidence obtained in several laboratories suggests that Ca^{2+} (from intracellular and extracellular pools) is necessary but not sufficient to mediate GnRH action (12–18).

It has recently been demonstrated that after its binding to its specific receptors, GnRH stimulates phosphoinositide turnover, mobilizes Ca^{2+}, activates protein kinase C (PKC), and induces arachidonic acid (AA) release (12). The production of multiple second messenger molecules is responsible for gonadotropin release and synthesis. Data will therefore be reviewed here showing that GnRH action includes at least seven steps in the mechanism rather than three as previously thought (9–11).

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I. Hormone-Receptor Interaction

Calcium-mobilizing receptors are thought to be members of the growing family of plasma membrane receptors that are linked to quanine nucleotide regulatory proteins (G proteins) (19–21). The family includes among other receptors, the α- and β-adrenergic, rhodopsin, muscarinic, cholinergic, LH, substance K, and pheromone receptors STE2 and STE3 of *Saccharomyces cerevisiae*. Common to this receptor family is that, after the ligand binding, receptor occupancy will dissociate the respective hetrotrimer G protein into its α- and $\beta + \gamma$-subunits. The free subunits (in most cases the Gα) will activate the effector system, which is an enzyme producing second messengers or an ion channel. Another common feature of the family is seven relatively conserved α-helical membrane-spanning domains connected to variable extracellular and cytoplasmic hydrophobic loops similar to the structure of rhodopsin (19–21).

The fifth and sixth hydrophobic membrane-spanning domains and the third cytoplasmic loop of the adrenergic receptor are involved in G protein interaction, whereas most of the hydrophobic domains (in particular the seventh) participate in conferring ligand binding specificity (20, 21). Since the GnRH receptor has not yet been cloned, knowledge on hormone-receptor interaction is derived only from binding studies, receptor purification, and photoaffinity labeling.

A. *Binding to the GnRH receptor*

The availability of a radioiodinated superagonist analog of GnRH modified at position 6 (D-amino acid) and a Fujino modification at the C terminus (des-Gly^{10}-*N*-ethylamide) enabled the establishment of a RRA for GnRH (22, 23). Stable analogs such as [D-Ser(t-Bu^6)] des-Gly^{10}-GnRH-*N*-ethylamide(GnRHa)] and others were used to characterize the binding sites in membrane preparations and in cultured pituitary cells (22–31). Once the RRA was established, binding studies were correlated with biological activity studies in the same cell cultures with the use of various GnRH analogs (32). Scatchard analysis of GnRHa binding to cultured rat pituitary cells revealed a single class of high-affinity sites [association constant (K_a) = 2.5×10^9 M^{-1}] that are specific for GnRH (27, 30). Occupancy of about 20% of GnRH binding sites is sufficient to obtain 80% of maximal LH release, indicating nonlinearity of GnRH receptor coupling to secretory responses ("spare receptors") in the gonadotropes (27). Calcium ions are not required for the first step of GnRH interaction with its receptors (27, 33). Formation of the hormone-receptor complex might be achieved by interaction of the positively charged arginine in position 8 of GnRH with negatively charged carboxyl groups in the binding sites of the receptor. Additional forces might be exerted by aromatic group interaction between the respective amino acids of both the hormone and the receptor (34, 35).

The dissociation constants (K_d) measured at 37 C were closely correlated with the biological potencies for several GnRH analogs examined (32). Comparative studies on binding affinity and *in vitro* biological potency for various GnRH analogs indicated that the binding sites measured indeed represent the hormone receptor that mediates the biological response.

B. *Regulation of GnRH receptors*

Changes in GnRH receptor number and cell responsiveness were observed during rat estrous cycle, pregnancy, and lactation (23, 36). Changes in the number of GnRH receptors were also noticed after castration, hypothalamic lesions, or after administration of GnRH antagonists or antibodies or an opioid agonist and antagonist (23, 36, 37). The changes in GnRH receptors indicated that GnRH regulates the maintenance of its own receptors. This was also supported by *in vitro* studies that indicated that GnRH regulates the number of its own receptors (up- and down-regulation) (38–40). Initially, receptor activation results in a loss of GnRH binding sites followed by a protein synthesis-sensitive process that culminates in an increase in receptor number when near physiological concentrations of GnRH are used (39). Interestingly, addition of the tumor promoter phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), at concentrations which activate pituitary PKC, to cultured rat pituitary cells resulted in up-regulation of GnRH receptors (41, 42). The finding is surprising in view of results in other systems in which TPA was reported to down-regulate hormone receptors (43, 44). Analysis of phosphorylation sites induced by PKC in epidermal growth factor (EGF) and in interleukin 2 receptors revealed that the phosphorylated residue was located in the putative cytoplasmic domain of the receptor, within 10 residues of the plasma membrane (45, 46). It was suggested that phosphorylation mediates the internalization process, which will ultimately lead to down-regulation (47–49). It is therefore surprising that TPA up-regulates GnRH receptors. We speculate that if PKC phosphorylates the GnRH receptor, the residues involved will not be in a juxtamembrane domain.

Pituitary gonadotrophs might differ from other cells since GnRH is known to up-regulate its own receptors both *in vitro* and during the reproductive cycle *in vivo* (23, 36–40, 50) as well as to activate PKC (51–53). It is therefore possible that PKC mediates the homologous up-regulation of GnRH receptors (41, 42). Indeed, the stimulatory effect elicited by TPA (55% increase in GnRH binding sites) is in excellent agreement with the

previously reported effect exerted by low concentrations of GnRH (39).

On the other hand, high concentrations of GnRH cause down-regulation of GnRH receptors, most likely due to massive internalization and processing of the hormone-receptor complex (38–40, 54, 55).

Changes in receptor number might be associated with target cell sensitivity in the face of variations of hormone concentrations (56, 57). Continuous exposure of pituitary cells to GnRH or its agonists, but not antagonists, resulted in desensitization of gonadotrope responsiveness to further stimulation by the hormone (58–60). The refractory state induced by high concentrations of GnRH was accompanied by down-regulation of GnRH receptors. The relative contribution of down-regulation of GnRH receptors, and the uncoupling of signal transduction units to desensitization, awaits further investigation (38–40, 58–62).

C. *Identification of GnRH receptors*

The use of a photoaffinity labeled GnRH analog enabled the identification of a 60 kilodalton (kDa) protein in pituitary membranes, presumably the GnRH receptor (63, 64). Using a ligand-immunoblotting technique, Eidne *et al.* (65) identified a similar binding component. GnRH receptors are most likely an heterogenous population with differences in the degree of glycosylation and hence the slight differences in mol wt of the binding components in the various reports. Higher mol wt estimates for the GnRH receptor were reported by others using either solubilization of the receptor (66–69), or inactivation of ionizing radiation (70).

The photoaffinity analog was also used to identify rat gonadal and human placental GnRH receptors that were shown to be similar to the pituitary receptors (64, 71–73). Whether the extra pituitary actions of GnRH have a physiological significance is not clear (74–78). Rat pituitary GnRH receptors were recently solubilized and purified (79, 80). Two components of 59- and 57 kDa were identified in good agreement with the photoaffinity labeling studies. The availability of a purified receptor preparation has enabled the development of monoclonal antibodies (81) and will enable partial amino acid sequencing of digested peptides, preparation of oligonucleotide probes, and, hopefully, the isolation of the GnRH-receptor gene.

D. *Localization of pituitary GnRH receptors*

With the use of morphological and biochemical approaches GnRH binding sites were shown to reside exclusively in the gonadotrophs (12, 82). Early studies by Hopkins and Gregory (83) utilized ferritin-labeled GnRH and showed that the peptide was internalized within 10 min after exposure.

With the use of video-intensified fluorescent microscopy and rhodamine-labeled (D-lys^6)-GnRH analog it was shown that receptor-bound GnRH is homogenously distributed on the surface of the gonadotropes followed by clustering and internalization of the hormone-receptor complex (Fig. 1 and Refs. 84 and 85). Light microscopy studies by Duello *et al.* (86) showed that the inter-

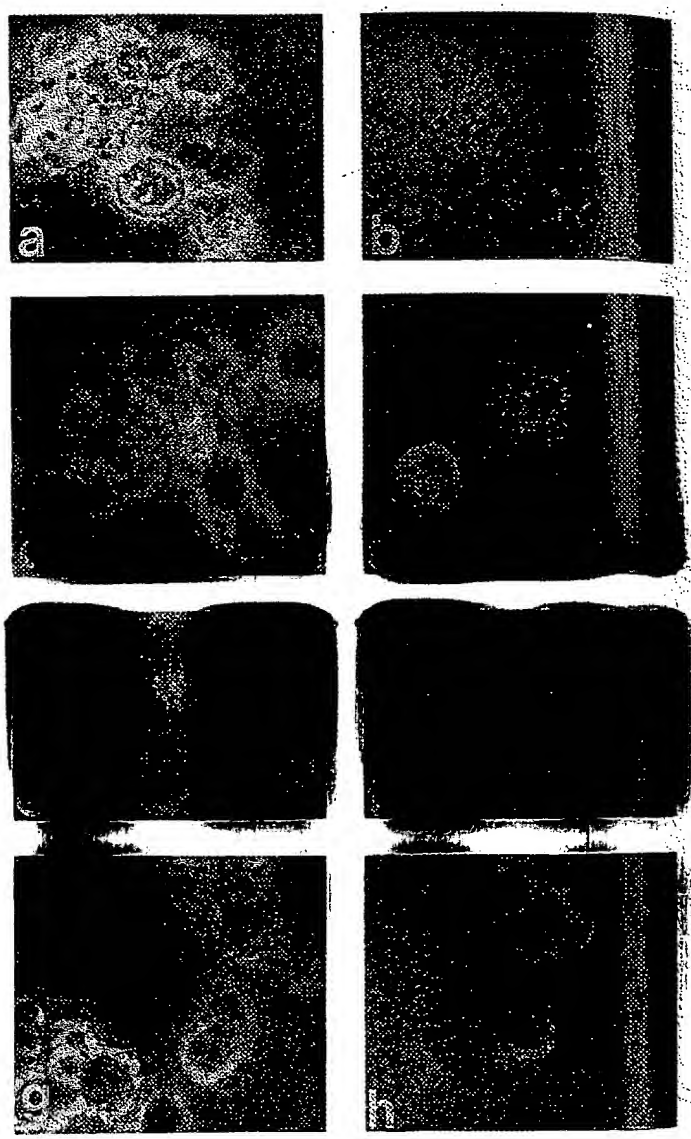

FIG. 1. Visualization of the GnRH-receptor interaction. Cultured pituitary cells were viewed alternately by phase contrast (*left*) or episcopic fluorescence microscopy (*right*). No fluorescence was observed in the presence of a 100-fold excess of (D-Lys^6]-GnRH (panels a and b). Uniformly distributed fluorescence was observed after 10 min of incubation in the presence of 10^{-7} M rhodamine-GnRH (panels c and d), in particular in the largest cells in the field. After 20 min of incubation, fluorescent clusters began to appear (panels e and f), and apparent internalization was observed after 30 min (panels g and h). [Reproduced with permission from Z. Naor *et al.*: *J Biol Chem* 256:3049, 1981 (85).]

nalization kinetics varied with the potency of the analog. Internalization of the labeled GnRH was seen as early as 5 min after exposure; by 10 min the label was seen over the Golgi complex and lysosomes. The kinetics of internalization agreed with those published by others (87–89).

GnRH is internalized by pituitary gonadotrophs in small vesicles, and the labeling is then found in organelles in the Golgi complex and lysosomes (86, 90). Differential processing of GnRH agonist and antagonist analogs was observed (91–94), suggesting that receptor activation might dictate the endocytotic pathway. Thus GnRH internalization follows the classical "receptor-mediated internalization pathway" as described for other peptide hormones (95). The pathway is used either for degradation of the ligand or release of the ligand from its receptor before recycling of the receptor (95). Labeling in the Golgi complex region may signify the binding of ligand to receptors that are being shuttled or recycled to the cell surface, or it may signify control of packaging of the hormone.

Using the avidin-biotin-peroxidase technique a D-lys^6-GnRH biotin (bio-GnRH) was prepared, and its binding to enriched fractions of gonadotrophs separated by centrifugal elutriation was investigated (96, 97). GnRH binding to the gonadotrophs was followed by the appearance of cellular processes or zones that stained intensely for bio-GnRH and the gonadotropins.

This apparent regional association of the hormones suggested that the site of receptor-mediated endocytosis and the site of exocytosis were linked geographically immediately after the stimulation. Formation of the processes may have occurred as a result of the addition of membranes by the secretory granules. Since the membranes may contain GnRH receptors, exocytosis of the granule may also have added more receptors for further binding and stimulation (12, 96, 97).

The percentage of cells labeled for Bio-GnRH matched the total percentage of gonadotrophs (~16%). It seems that GnRH binds to the multihormonal cells (gonadotrophs containing LH + FSH cells, ~57% of total gonadotrophs) as well as to the monohormonal cells (LH cells, ~18%; FSH cells, ~22%) (12, 98). Studies with the Bio-GnRH probe confirmed earlier observations of GnRH staining on the secretory granules (99–101), suggesting that secretory granules may provide a vehicle for receptor recycling during GnRH stimulus and also provide a mechanism for homologous up-regulation.

In summary, GnRH binds to and activates only the gonadotrophs. Following the initial binding the GnRH receptor complex forms patches and is internalized in small vesicles. At the same time gonadotropin stores are mobilized to the site of endocytosis of the ligand-receptor complex. The endocytotic vesicles may transport GnRH to lysosomes for degradation, while multivesicular bodies and possibly secretory granules may be used to recycle GnRH receptors back to the surface.

II. Receptor-GTP-Binding Proteins Interaction

Guanine nucleotide binding proteins (G proteins) are heterotrimeric proteins located on the cytoplasmic surface of cell membranes. G Proteins serve as transducers to couple the receptor and the effector units. They belong to a large family of GTP-binding proteins that includes major groups such as elongation factors (EF-TU), G proteins (*e.g.* G_s, G_i, G_t, G_o) and the low mol wt GTP-binding proteins (*e.g. ras* protein) (102–104). The G proteins are involved in light-activated photoreceptors (G_t, transducin), in adenylate cyclase regulation (G_s, G_i) and neuronal ion channel conductance (G_o). For some putative G proteins a function was found before the protein was identified, *e.g.* G_p for phospholipase C (PLC) activation; G_k for ion channel conductance, G_e for exocytosis, and a G protein involved in phospholipase A_2 activation (102–108). On the other hand, some G proteins were already isolated, but their function is not known [several forms of G_i and G_t (109)]. A family of low mol wt G proteins (19–27 kDa) also known as *ras*-like proteins was also described (103). This family includes the three c-*ras* gene products involved in transformation, low mol wt GTP-binding proteins from yeast such as Ypt1p and Sec4p which might serve as labels for individual secretory vesicles, and the mammalian low mol wt G proteins whose functions are not known yet (103). ADP-ribosylating toxins interact with various members of the G proteins. G_s and G_t are ADP-ribosylated by cholera toxin, while G_{i1-3}, G_o, and G_t are ADP-ribosylated by pertussis toxin (102).

The G proteins are heterotrimers consisting of α, β, and γ-subunits. Whereas the α-subunit (39–54 kDa) is distinct among the various G proteins, the β (35–36 kDa) and the γ (8–10 kDa) are apparently similar. Under basal conditions, the α-subunit is bound to GDP and the heterotrimer is intact. After binding of a ligand to its specific receptor, GDP is replaced by GTP, and the complex dissociates to α and $\beta\gamma$. The free α-GTP modulates the effector activity (*e.g.* adenylate cyclase). Intrinsic GTPase activity converts the GTP to GDP, resulting in deactivation of the process and reassociation of the subunits (102, 110–112).

A novel G protein-mediated signalling pathway was recently described (104, 113, 114). It was claimed that upon ligand binding the $\beta\gamma$-complex activates membrane-bound phospholipase A_2 resulting in the formation of AA and lipoxygenase products which in turn are capable of activating myocyte muscarinic K^+ channels (104, 113, 114). The $\beta\gamma$-subunits of transducin have been

shown to activate phospholipase A_2 in rod outer segments (108). Also the $\beta\gamma$ of yeast G protein are involved in mating behavior in *Saccharomyces cerevisiae* (115). Lipoxygenase products of AA were implicated in opening of K^+ channels in Aplysia sensory neurons (116, 117). The above reports support a $\beta\gamma$-phospholipase A_2 signalling pathway.

Evidence that the GnRH receptor is coupled to a G protein emerged only recently when it was demonstrated that nonhydrolyzable analogs of GTP inhibit the binding of GnRH agonists but not antagonists in bovine and rat anterior pituitary membranes (118, 119). The data are consistent with modulation by guanine nucleotides of agonist binding to G protein-coupled receptors (19, 120). Further support to the above claim is derived from observations that introduction of GTP analogs to permeabilized pituitary cells resulted in enhanced inositol phosphate formation and LH release (119, 121). Furthermore, Andrews *et al.* (121) reported inhibition of the GTP effect by a GnRH-antagonist limiting the response to the gonadotropic cells. Further studies are needed to characterize the G proteins coupled to the GnRH-receptor and their role in signal transduction.

III. Activation of Phosphoinositide Turnover

Hokin and Hokin (122) were the first to suggest that phosphatidylinositol (PI) turnover might play a role in the muscarinic cholinergic response. It was not until 1975 that Michell (123) proposed that PI turnover represents a more general mechanism for signal transduction for Ca^{2+}-mobilizing receptors. In 1978 Akhtar and Abdel-Latif (124) demonstrated that acetylcholine increased the breakdown of ^{32}P-labeled phosphatidylinositol 4,5-bisphosphate (PIP_2). Since the effect was Ca^{2+} dependent, the recognition that PIP_2 hydrolysis might trigger Ca^{2+} mobilization did not come for another 7 yr (Ref. 125 for review). It was then that attention was drawn to the importance of the polyphosphoinositides (125, 126). It is presently thought that the first substrate to be acted upon by Ca^{2+}-mobilizing ligands (~120 such ligands have been described) is PIP_2 (Fig. 2). The activation of PLC is most likely mediated by a GTP-binding protein (G_p) analogous to the role of G_s in the adenylate cyclase system (102, 105–107, 111, 112, 125, 126). Whereas in some cell types such as neutrophils, macrophages, lymphocytes and fibroblasts, the phosphodiester cleavage is pertussis toxin-sensitive, in other cells such as brown adipocytes, astrocytomas, GH_3, pituitary gonadotrophs, and pancreas, liver, and heart cells, the activation process is insensitive to the toxin (105–107, 112, 125–128). It is therefore thought that at least two different but related G proteins (G_i, which is inactivated by pertussis toxin, and G_p) mediate the hydrolysis of PIP_2 in the respective cell type. A potential candidate to serve as G_p is G_z, which was recently cloned by reduced stringency with transducin α-complementary DNA (cDNA) (109). The consensus sequence Cys-Gly-Leu (Phe- or Tyr) in which the cysteine is ADP-ribosylated is missing in $G_{z\alpha}$; hence G_z might be the pertussis toxin-insensitive G_p.

Thus receptor-mediated activation of PLC is thought to be mediated by the coupling of the receptor and a G protein. This was confirmed in numerous studies in whole cells, permeable cells, and membrane preparations (102, 105–107, 127, 128). Interestingly, activation of PLC by platelet-derived growth factor in a rat fibroblast cell line (WFB) does not seem to be coupled via a G protein (129). It was therefore theorized that receptors with seven trans membrane spanning segments activate PLC via a G protein while those with only a single membrane-spanning segment (*e.g.* platelet-derived growth factor, EGF) activate PLC by a unique mechanism not involving a stimulatory G protein (G_p) (129). Indeed recent studies have shown EGF-induced tyrosine phosphorylation of PLC II (130, 131), suggesting direct activation of the effector by the receptor.

A specific PLC acting at resting Ca^{2+} levels hydrolyzes PIP_2 to generate DG and myo-inositol-D-1,4,5-trisphosphate (IP_3). DG and IP_3 are now considered second messengers since IP_3 mobilizes intracellular Ca^{2+} (125, 126), by binding to specific receptors (132–134), and DG activates PKC (135, 136).

Initially, soluble PLC was thought to act only on PI but later it was recognized that soluble PLC hydrolyzes all three phosphoinositides (PI, phosphatidylinositol 4-phosphate (PIP), and PIP_2) albeit with different Ca^{2+} sensitivity (137). Presentation of the membrane substrate to the enzyme might occur by membrane perturbation, and arachidonate might play a role in this process (138). Membrane-bound PLC activity was also described as capable of activating the direct hydrolysis of PIP_2 and possibly PIP but not PI (139).

The first substrate to be acted upon during enhanced PI turnover is controversial. Berridge (125, 126) has suggested that it is PIP_2 and hence IP_2 and IP are derived from IP_3. On the other hand, Majerus' group (140) claims that initially PIP_2 and PIP are hydrolyzed followed by a massive hydrolysis (up to 50%) of PI. A similar conclusion was reached by Imai and Gershengorn (141). The practical difference between the two theories is that according to Berridge, IP_3 and DG will be produced simultaneously, and therefore Ca^{2+} mobilization and PKC activation occur in parallel. However, according to the second hypothesis, DG might be produced from PI at a later step in hormone action, and cellular activation could proceed sequentially in such a way that cellular

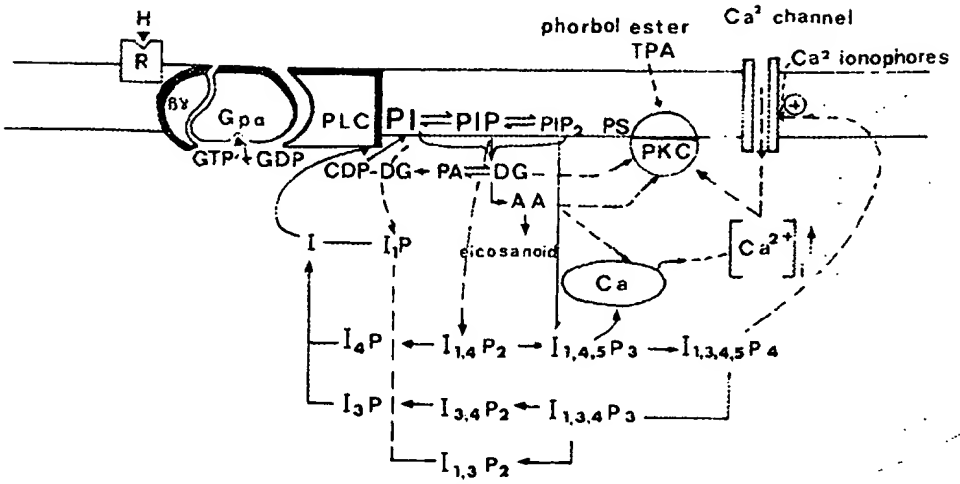

FIG. 2. Phosphoinositide turnover. Hormone (H) receptor (R) activation results apparently in dissociation of G_p and activation of PLC. Hydrolysis of PIP_2 by PLC results in the formation of DG and inositol 1,4,5-trisphosphate (I 1,4,5 P_3,IP_3). DG can also be formed from hydrolysis of PIP or from PI. DG is converted to PA and further to cytosine diphosphate-DG. An exchange with free inositol (I) forms PI, and multiple phosphorylation produces first PIP and later PIP_2. DG can also be acted upon by DG- and monoacylglycerol lipase to form AA which is further metabolized to eicosanoids (prostaglandins, thromboxane, and leukotrienes). IP_3 can mobilize cellular Ca^{2+} pools, and elevated Ca^{2+} and DG will translocate and activate PKC, which will bind in the membrane to DG and PS. I(1,4,5)P_3 is metabolized to I(1,3,4,5)P_4 which is thought now to be involved in Ca^{2+} influx. IP_4 is then converted to the inactive IP_3 isomer I(1,3,4)P_3 which is further metabolized to I(3,4)P_2, further to I(3)P and to free inositol (I). I(1,3,4)P_3 can also be converted to I(1,3)P_2, further to I(1)P and I. The active isomer of IP_3 namely I(1,4,5)P_3 is alternatively degraded to I(1,4)P_2, further to I(4)P and free I. The direct formation of I(1,4)P_2 and I(1)P from PIP and PI, respectively, is also shown here by a *broken line*, but this route is not yet clear.

Ca^{2+} might be responsible for a transient effect, whereas PKC might participate in the sustained hormone action.

It is therefore possible that initially only the membrane-bound PLC is activated by the occupied receptor via G_p leading to hydrolysis of PIP_2 and PIP. The rapid formation of IP_3 will induce a Ca^{2+} transient that might be sufficient to trigger a burst phase of exocytosis (142, 143). The Ca^{2+} rise might also present the membrane-bound PI to hydrolysis by soluble PLC. This will generate the bulk of DG needed for PKC activation (140, 141). Sequential hydrolysis of phosphoinositides might regulate the timing and degree of activation of the bifurcating Ca^{2+}-messenger system.

Recent attempts to purify the enzymes have shown the existence of three immunologically distinct forms of PLC [types I, II, and III (144, 145)]. Whereas PLC-II is exclusively cytosolic, PLC-I was evenly distributed between the cytosol and the particulate fraction. PIP and PIP_2 were found to be better substrates as compared to PI for both PLC-I and PLC-II when Ca^{2+} concentration was fixed at the micromolar range (144, 145).

PLC-I (150 kDa) contains catalytically active 140- and 100-kDa forms and an inactive 41-kDa protein (144, 145). It is attractive to speculate that this 41-kDa protein might be a G protein. Indeed, Deckmyn *et al.* (146) proposed that PLC and a G protein might exist in the cytosol. The availability of various monoclonal antibodies to PLC-I might be used to elucidate the nature of G_p.

The first sequence of a mammalian PI-PLC was recently reported (145, 147). Homology was noticed with noncatalytic domains of the nonreceptor class of tyrosine kinase (*e.g. yes, fgr, fps, src*) and $p47^{gag\text{-}crk}$, a gag-fusion polypeptide produced by the avian sarcoma virus CT10 (148). Other sequences of homology, known as B,C boxes, were found between PLC-II and the GTPase activating protein (GAP) which is thought to be involved in the action of the *ras* encoded p21 protein (149, 150). As in the case of phorbol esters, this is another clue to a possible involvement of PI turnover-generated second messenger molecules in transformation. The observation that PLC and nonreceptor tyrosine kinases contain homologous regions raises the interesting possibility of mutual regulatory effectors. Indeed EGF phosphorylates PLC-II via tyrosine kinase (130, 131). More recently a PI-PLC isozyme was cloned and was found to have about 55% homology with thioredoxin, a protein cofactor in thiol-dependent redox reactions (151). This surprising finding suggests a possible labile inositide-phosphothiol intermediate during the phosphodiesteric cleavage of polyphosphoinositides.

DG is phosphorylated to phosphatidic acid (PA) or else acted upon by DG lipase to form AA. PA is then converted back to PI, and multiple phosphorylation of PI will produce PIP and PIP_2. PA was regarded for a while as a Ca^{2+} ionophore (152), but this role was not confirmed. It was recently suggested that PA might be involved in cell proliferation (153) and might also interfere with the recently described GTPase activating pro-

tein [GAP (150)]. Hence PA might affect the activation state of certain G proteins.

The complex metabolism of IP_3 was recently investigated (154). An inositol phosphate 5-phosphomonoesterase converts inositol 1,4,5-trisphosphate [I (1,4,5)P_3] to I(1,4)P_2 which is then hydrolyzed further by inositol polyphosphate 1-phosphatase to I(4)P and finally to inositol (155, 156). I(1,4,5)P_3 is also metabolized to I(1,3,4,5)P_4 by a 3-kinase in a Ca^{2+}-dependent manner (157, 158). Thereafter, IP_4 is converted to the IP_3 isomer I(1,3,4)P_3 by inositol phosphate 5-phosphomonoesterase. No biological role has yet been attributed to I(1,3,4)P_3 which is hydrolyzed to I(3,4)P_2, further to I(3)P, and finally to inositol (Refs. 154–158 and Fig. 2). It is possible therefore that previous reports indicating Ca^{2+}-independent hydrolysis of polyphosphoinositides were measuring mainly I(1,4,5)P_3, while those reporting Ca^{2+}-dependency were measuring I(1,3,4)P_3, which requires Ca^{2+} for its two-step isomerization. Indeed anion exchange chromatography techniques as used by most investigators cannot differentiate between the two isomers, which are believed to be produced sequentially: first I(1,4,5)P_3 and later I(1,3,4)P_3. It is now possible to measure the formation of I(4)P *vs.* I(1)P using HPLC methods. Therefore it was assumed for a while that if ligand stimulation produces I(4)P, it might have come from PIP_2 or PIP and if the major product was I(1)P then the major phospholipid to be hydrolyzed was PI. Although it seems a subtle difference, it is very significant. Since DG will also be liberated, the timing and the amount of DG released will be determined by whether the PLC substrate was PIP_2, PIP, or PI. Since PIP_2 is a very minor phospholipid (~1% of total phospholipid) its rapid hydrolysis will supply a small and transient amount of DG for PKC activation. On the other hand, if PI is hydrolyzed, more DG will be released over a longer period of time, allowing a more sustained activation of PKC. As previously mentioned, whereas it is generally accepted that PIP_2 is the substrate for PLC (125, 126) recent studies have suggested that although PIP_2 is hydrolyzed initially it is followed by hydrolysis of PI (140, 141). Whether this is the case in other systems is not yet known and should be investigated.

The metabolism of IP_3 now seems to have been made even more complex by the finding that I(1,3,4)P_3 can also be converted to I(1,3)P_2 and further to I(1)P (159). Thus the presence of I(1)P *vs.* I(4)P in a given biological system cannot differentiate the PLC substrate since both can be derived from PIP_2 (159). To add another complexity to the field, it was reported that the formation of the various inositol phosphates is accompanied by the respective production of inositol cyclic phosphates (ester linkage between carbons 1 and 2 of the respective IP, IP_2, and IP_3) (160–162). The role of the inositol cyclic phosphates is still not known. I(1,2cyc4,5)P_3 was found to be as effective as I(1,4,5)P_3 in mobilizing intracellular Ca^{2+} (161). Whether this cyclic inositol phosphate is formed in cells should be further clarified.

The important role of I(1,4,5)P_3 in mobilizing intracellular Ca^{2+} is well established (125, 126, 133, 158). Its formation is usually rapid and transient and is followed later by the appearance of the more stable isomer I(1,3,4)P_3 (155, 163). Although it was previously suggested that I(1,4,5)P_3 was the only biologically active inositol phosphate, recent studies suggest that I(1,3,4)P_3 is also capable of mobilizing intracellular Ca^{2+} albeit with less efficacy [ED_{50} 9 μM *vs* 0.3 μM for I(1,4,5)P_3] (164). A role for I(1,3,4,5)P_4 in enhancing Ca^{2+} influx from extracellular sources in sea urchin eggs was suggested (158, 165). The effects of IP_4 is absolutely dependent on the presence of IP_3, and both were shown to stimulate Ca^{2+}-induced K^+ channel activation in patch clamps, an indication of Ca^{2+} entry (158, 166). It is possible that Ca^{2+} mobilization by IP_3 is necessary to enable Ca^{2+} influx by IP_4 for refilling the intracellular pools as theorized by Putney (167). According to the "capacitative Ca^{2+} entry" theory when the IP_3-sensitive Ca^{2+} pool is depleted, an unidentified mechanism is activated to refill this pool (167, 168). Whether IP_4 is "the other half of the story" as suggested by Irvine's group (158, 166) needs to be confirmed. High affinity binding sites for IP_3 were demonstrated in adrenal microsomes (133), hepatocytes, neutrophils (132), and brain (134). It was noticed that GTP increased the amount of Ca^{2+} released by IP_3 in liver microsomal fractions (169). Is there another protein mediating the effect of IP_3 on Ca^{2+} mobilization? If this was the case, then one would predict that a nonhydrolyzable analog of GTP such as guanosine 5′-(β,γ-imido) triphosphate would be more effective in sustaining Ca^{2+} mobilization compared to GTP. However, Ueda *et al.* (170) found no Ca^{2+} mobilization by guanosine 5′-(β,γ-imido)triphosphate in N1E-115 neuroblastoma cells, indicating that GTP hydrolysis is required. Whether GTP is obligatory for IP_3 action is not clear, but a G protein does not seem to be involved in IP_3 action.

During PI turnover DG is phosphorylated to PA. PA is then converted back to PI, and multiple phosphorylation of PI will produce PIP and PIP_2. On the other hand, the IP_3 is converted to inositol, and PI is resynthesized from inositol and activated DG. Thus the importance of the early phosphoinositide response is the generation of the "second messengers" DG and IP_3, (and possibly IP) which are involved in PKC activation and Ca^{2+} mobilization, respectively (125, 126, 133, 137–140).

A. Phosphoinositide turnover and GnRH action

Involvement of phospholipid turnover in GnRH action was first demonstrated in 1981 (171). Enhanced PI turn-

over by GnRH was then demonstrated in pituitary cells (172–176), in granulosa cells (177–179), and in testicular cells (180). However the early studies have not identified PIP_2 as the first substrate of GnRH action on PLC activity in the pituitary. More recently the rapid hydrolysis of pituitary PIP_2 by GnRH was demonstrated (128, 181, 182). Addition of GnRH to pituitary cells prelabeled with myo-[2-^{3}H]inositol stimulated the appearance of labeled IP_3 (~10 sec) in the presence of Li^+ in the medium (Ref. 128 and Fig. 3). Morgan *et al.* (182) identified the IP_3 as I(1,4,5)P_3 and also found higher polyphosphoinositols (IP_4, IP_5, and IP_6) after GnRH challenge. Activation of PLC by GnRH does not require elevation of cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) and therefore might precede the mobilization of cellular Ca^{2+} by GnRH (128). Activation of PLC by GnRH is not inhibited by pertussis toxin, suggesting that the GTP-binding protein involved in GnRH action is not G_i or G_o but more likely G_p (119, 128).

Permeabilization of pituitary cells enabled introduction of GTP and IP_3 in order to examine their role during PI turnover. Addition of GTP or guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γS) to ATP^{4-}-permeabilized pituitary cells resulted in enhanced PLC activity as determined by inositol phosphate (Ins-P) production and gonadotropin secretion (119, 121). The effect of GTP-γS on Ins-P production was not additive with that of GnRH, but the combined effect was partially inhibited by guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) which maintains G proteins in the inactive state, or by neomycin, a PLC inhibitor (119). The above results and the finding that pretreatment of pituitary membranes with GTP or GTP-γS resulted in reduction in GnRH binding affinity (118, 119) suggested the involvement of a G protein (G_p) in the stimulus-coupling mechanism of GnRH action.

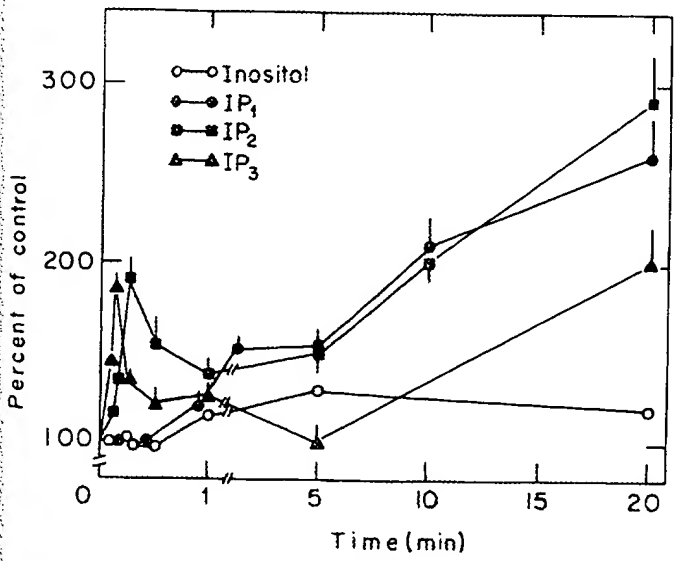

FIG. 3. Time course of the effect of GnRH on the levels of [^{3}H]Ins-P. Cultured pituitary cells (5×10^6 cells per dish) were prelabeled with myo-[2-^{3}H]inositol for 3 days. Cells were then treated shortly (15 min) with Li^+ (10 mM) and finally with or without GnRH (100 nM) for the time indicated. Ins-P were extracted and separated on Dowex 1×10 columns. The results shown are mean ± SE expressed as percent of control for each time point. [Reproduced with permission from Z. Naor *et al.*: *J Biol Chem* 261:12506, 1986 (128).]

The claim that GnRH mobilizes an intracellular Ca^{2+} pool (183) was strengthened by recent findings that IP_3 can mobilize cellular Ca^{2+} in cultured pituitary cells (17) as demonstrated in other cell systems (125, 126, 184, 185). In another approach, IP_3 receptors were described in pituitary membranes ($K_d = 1.1 \pm 0.4$ nM; maximal binding 28 ± 15 fmol/mg protein) and the Ca^{2+} releasing activity of IP_3 was demonstrated in pituitary membrane preparations (186). Thus one of the earliest effects induced by GnRH in pituitary gonadotrophs is a rapid Ca^{2+}-independent phosphodiesteric hydrolysis of phosphoinositides to generate the second messengers IP_3 and DG required for further processing of the Ca^{2+} evoked signal.

IV. Phospholipase D

Phospholipase D (PLD) catalyzes hydrolytic cleavage of the terminal diester bond of phospholipids, resulting in the direct formation of PA and the respective base (187–189). Although the enzyme was characterized mainly in plants, recent studies have shown similar enzymatic activities in mammalian cells (187–193). The unique ability of PLD to catalyze transphosphatidylation in which the phosphatidyl group can be transferred to a nucleophile, such as ethanol, producing phosphatidylethanol, enabled a reliable enzymatic assay. Receptor-activated reaction was demonstrated for *N*-formyl-Met-Leu-Phe-stimulated neutrophils and HL-60 granulocytes (188–191), for vasopressin-stimulated hepatocytes (189), and for GnRH agonist-stimulated granulosa cells (193).

The importance of PLD in signal transduction can be exerted at two levels. The formation of PA might be important for Ca^{2+} regulation (152), for cell proliferation (153), and for inhibition of the recently described GTPase activating protein (GAP) (150). On the other hand PA can be converted to DG by PA phosphohydrolase and therefore participate indirectly in PKC activation. Interestingly, most of the PA and DG formed during activation of HL-60 cells by *N*-formyl-Met-Leu-Phe is the result of PLD activation (191). Hence regulation of PKC activity in this case might be dictated mainly by PLD. On the other hand PLD itself is activated by Ca^{2+}, DG, and TPA (191, 192). It is therefore possible that initially PLC activates PKC (by hydrolysis of PIs) and supplies Ca^{2+} and DG needed for PLD activation, which in turn will act on other phospholipids such as phospha-

tidylcholine to produce the bulk of DG needed for sustained PKC activation.

The recent finding that a GnRH agonist stimulates PLD activity in rat granulosa cells (193) raises the possibility that similar findings might be observed at the pituitary level since a great similarity exists between GnRH action at the pituitary and gonadal level (74–77, 177–180).

Does nature always segregate the various units of the signal-transduction apparatus so that the receptor-transducer (G proteins) and the effector (adenylate cyclase, PLC, PLD) are separate entities? It seems that this is the case in most of the systems studied. However, the case of guanylate cyclase represents an interesting exception (194, 195). Membrane guanylate cyclase was recently found to be the receptor for atrial natriuretic peptide and for Resact, a chemotactic peptide released by sea urchin eggs (194, 195). Thus, receptor-transducer and effector can be found in one molecule.

Although GnRH can elevate both cAMP and cGMP under certain conditions, the cyclic nucleotides are not involved in the acute exocytotic response of the neurohormone (196–201). cAMP however might be involved in GnRH-induced gonadotropin biosynthesis (202–204).

V. Role of Calcium

The role of Ca^{2+} in stimulus-secretion coupling mechanisms is well documented (205–207). Recent interest in the field has focused on the molecular mechanisms involved in Ca^{2+} mobilization (125, 126, 133, 158). The recognition that IP_3 mobilizes cellular Ca^{2+} and that IP_3 and IP_4 might be involved in Ca^{2+} influx has been a key element in understanding the function of a Ca^{2+}-mobilizing receptor (125, 126, 133, 158). With the recent introduction of fluorescent Ca^{2+} probes such as Quin 2 and Fura 2, direct measurements of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in stimulated cells became the method of choice (208–212).

Various studies have indicated that the release of pituitary hormones in general, and of the gonadotropins in particular, is Ca^{2+}-dependent (9, 183, 213-224). A role for Ca^{2+} as a mediator of GnRH-induced gonadotropin release was therefore suggested. This was based on the observations that extracellular Ca^{2+} was required for GnRH-stimulated LH release; at high concentrations (100 μM) the Ca^{2+} ionophore A23187 mimicked GnRH action; Ca^{2+} channel blockers such as verapamil and La^{3+} inhibited GnRH-induced LH release; calmodulin antagonists blocked GnRH action, and GnRH induced a transient rise in $^{45}Ca^{2+}$ efflux concomitant with elevated release of LH (9, 213–222). However detailed analyses of the Ca^{2+} pools mobilized by GnRH, and direct measurements of changes in $[Ca^{2+}]_i$ induced by GnRH in the gonadotrophs have only recently been performed. Studies of this kind have been hampered so far by the fact that pituitary cells are heterogeneous, and the gonadotrophs constitute only about 10% of the total pituitary cell population (97, 225). The recent availability of a rapid and reliable method for the isolation of a gonadotroph enriched cell population by centrifugal elutriation (8, 97, 226) and the introduction of fluorescent probes such as Quin 2/AM and Fura 2/AM, which can be trapped in cells, permitting direct determination of $[Ca^{2+}]_i$ (208–212), enabled investigators to study the effect of GnRH on $[Ca^{2+}]_i$ in pituitary gonadotrophs. In the presence of extracellular Ca^{2+} GnRH induces a rapid Ca^{2+} transient reaching maximal levels within about 8–10 sec (160% of basal, first phase) which is followed by a slow and gradual decay of $[Ca^{2+}]_i$ which lasts for about 20 min (second phase, Fig. 4, and Refs. 17 and 183). Analysis of the Quin-2 fluorescence from many experiments showed that pituitary gonadotrophs basal $[Ca^{2+}]_i$ was about 150 nM, and this level was elevated by about 50–100 nM by GnRH (17, 183, 223, 224). This effect of GnRH was observed by several investigators and is remarkably small when compared, for example, to the effect of TRH on $[Ca^{2+}]_i$ in GH_3 cells (227, 228).

The rise in $[Ca^{2+}]_i$ stimulated by GnRH did not reach the 500 nM range and therefore remained below the concentration generally needed for a $[Ca^{2+}]_i$ rise to elicit secretion by itself (229, 230). It was therefore suggested that Ca^{2+} is not sufficient to mediate GnRH effect on gonadotropin secretion (17). When Ca^{2+} was removed from the medium by EGTA and GnRH was introduced to pituitary gonadotrophs, similar rapid kinetics of elevated $[Ca^{2+}]_i$ was noted, but the effect was usually terminated within about 2 min (Fig. 4 and Refs. 17 and 183). The results were interpreted as an indication of Ca^{2+} mobilization from an intracellular pool by GnRH. This was in contrast to previous reports indicating that GnRH mobilizes only extracellular Ca^{2+} pools (9–11). To further support the proposal of an intracellular Ca^{2+} pool mobilized by GnRH, attempts were made to deplete this pool by low concentrations of ionomycin (227). Indeed, when GnRH was added after ionomycin in the absence of extracellular Ca^{2+} no rise in $[Ca^{2+}]_i$ was noted (Fig. 4 and Ref. 17). Therefore an intracellular ionomycin-sensitive Ca^{2+} pool is mobilized rapidly by GnRH and is most likely mediated by IP_3 formation. This was suggested by findings that the time course of IP_3 formation by GnRH (5–10 sec) was in agreement with the Ca^{2+} transient induced by GnRH in the gonadotrophs (~8 sec) (17, 128). Data were also presented to show that IP_3 can release Ca^{2+} from an intracellular nonmitochondrial organelle in pituitary cells (17), and IP_3 binding sites are present in pituitary microsomal preparations (186) suggesting that GnRH indeed mobilizes the IP_3-sensitive

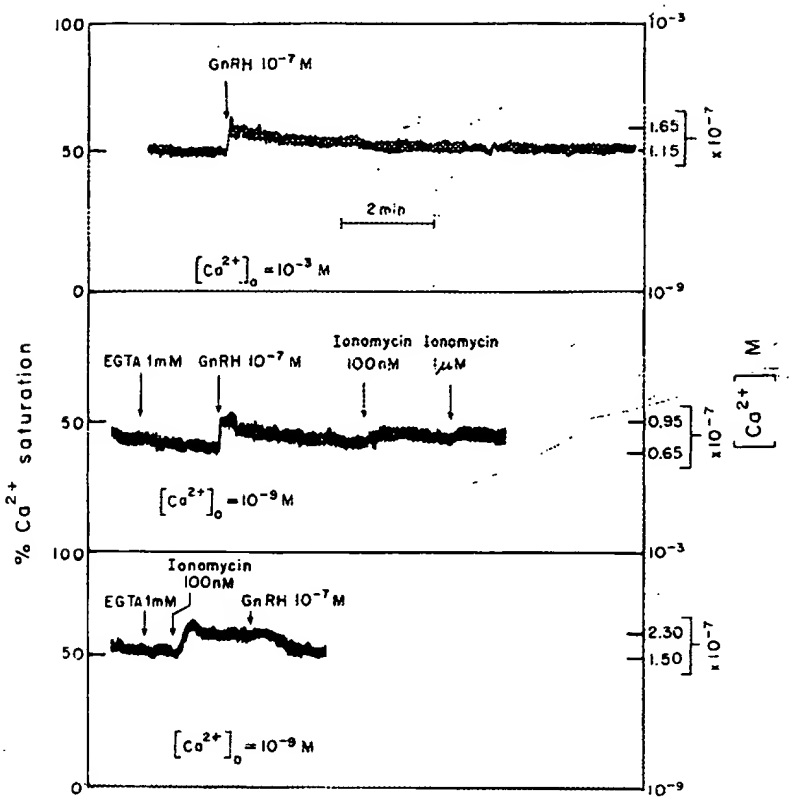

FIG. 4. GnRH-induced elevation of $[Ca^{2+}]_i$ in pituitary gonadotrophs separated by centrifugal elutriation. Cells were loaded with Quin 2/AM and transferred to normal medium (*upper panel*) or to nominally Ca^{2+}-free medium (*lower two panels*) before stimulation with GnRH and ionomycin. Traces of Quin-2 fluorescence of representative experiments are shown. Basal and stimulated levels of $[Ca^{2+}]_i$ are given. Additions were made at the times indicated by the *arrows*. [Reproduced with permission from Z. Naor *et al.*: *Mol Endocrinol* 2:512, 1988 (17). © The Endocrine Society.]

Ca^{2+} pool. To demonstrate the effect of IP_3, pituitary cells had to be permeabilized by high voltage electric field discharge and maintained in very low Ca^{2+}-free buffer. Ambient free Ca^{2+} concentration ($[Ca^{2+}]_c$) (at steady state $[Ca^{2+}]_c$ is equivalent to $[Ca^{2+}]_i$) monitored by Ca^{2+}-sensitive minielectrodes will be reduced from around 1 μm to a first steady state of around 500 nM, which is the buffering capacity of mitochondria. However in the presence of ATP and mitochondria inhibitors $[Ca^{2+}]_c$ will be reduced further to about 170 nM, which is close to gonadotroph $[Ca^{2+}]_i$, and believed to be the Ca^{2+} level maintained by the IP_3-sensitive nonmitochondrial pool which was described in other cell types (*e.g.* Refs. 184 and 185). Under these conditions addition of IP_3 prompted a transient rise in $[Ca^{2+}]_c$ consisting of efflux and reuptake most likely into an IP_3-insensitive Ca^{2+} pool as shown in adrenal glomerulosa and GH_3 cells (Fig. 5 and Refs. 184 and 185). The IP_3-releasable Ca^{2+} pool in pituitary cells was estimated at about 1.5 nmol/8 $\times$ 10^6 cells and represents most likely only a fraction of the total intracellular Ca^{2+} pool. The Ca^{2+} pool mobilized by GnRH accounts for about 55% of the peak $[Ca^{2+}]_i$ induced by GnRH; the other 45% of the peak $[Ca^{2+}]_i$ is derived via voltage-dependent Ca^{2+} channels. A common biochemical event might be responsible for cellular Ca^{2+} mobilization and initiation of Ca^{2+} influx. Indeed, it was suggested that Ca^{2+} mobilization by IP_3 might be followed by Ca^{2+} influx to replenish the IP_3-sensitive pool (167). Also, I(1,3,4,5)P_4 could serve as a mediator of Ca^{2+} entry after IP_3 action as previously discussed. This scheme might be relevant for pituitary gonadotrophs as GnRH was shown to elevate the level of I(1,3,4,5)P_4 (182).

The first transient phase of LH release induced by GnRH was shown to be insensitive to the Ca^{2+} channel blockers D600 and nifedipine (143). It is therefore possible that this rapid phase of secretion is initiated by the IP_3-sensitive intracellular Ca^{2+} pool. The existence of a significant component of GnRH-induced gonadotropin secretion that is not dependent on extracellular Ca^{2+} was also demonstrated in static and perifused pituitary cells (14, 17, 143). AA and/or its metabolites (AA/m) were implicated in GnRH action upon gonadotropin secretion (171, 231–235). Since AA-induced LH release is a Ca^{2+}-independent process (171, 236) it was recently suggested that AA and/or AA/m might participate in the initiation of LH secretion by GnRH and hence participate in this fraction of GnRH action, which is Ca^{2+}-independent (236).

The Ca^{2+}-independent fraction of the exocytotic response to GnRH might also be mediated by a G-protein specializing in exocytosis. Indeed introduction of GTP-γS to permeabilized pituitary cells resulted in enhanced LH release that amounted to about 30% of the exocytotic response elicited by GnRH in resealed cells (119). The stimulatory effect of GTP-γS, unlike that of GnRH, was

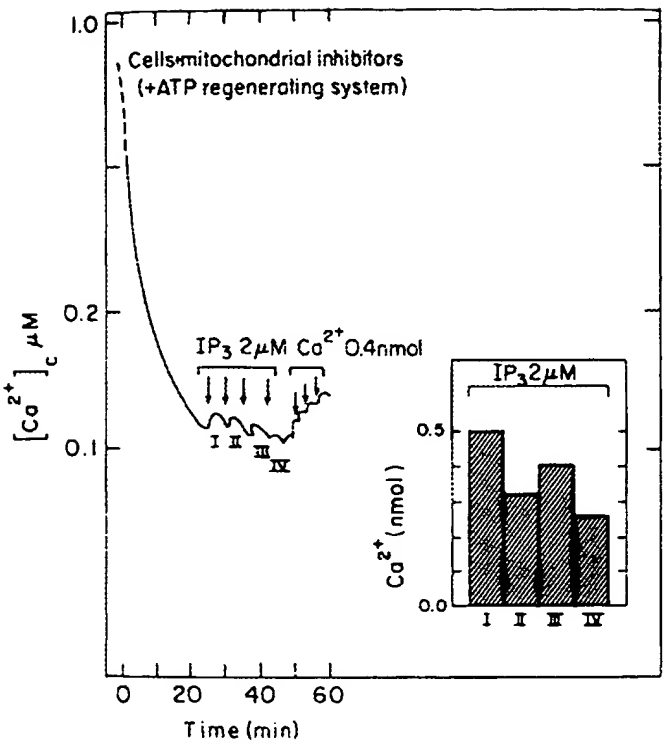

FIG. 5. Effect of IP_3 on Ca^{2+} release from permeabilized pituitary cells. Pituitary cells were permeabilized using a high voltage electric field discharge. $[Ca^{2+}]_c$ was measured using Ca^{2+} selective minielectrodes. The cells were incubated in a buffer containing mitochondrial inhibitors and an ATP regenerating system. Ca^{2+} uptake was initiated by the addition of the cells. Ca^{2+} release was calibrated by comparison with successive additions of known standards of $CaCl_2$. [Reproduced with permission from Z. Naor *et al.*: *Mol Endocrinol* 2:512, 1988 (17). © The Endocrine Society.]

TABLE 1. Effect of GTPγS on LH release

Treatment	LH released (ng/ml)	
	EGTA (3 mM)	Ca^{2+} (1 mM)
Control	17.5 ± 3	13.3 ± 3
GTPγS (100 μM)	38.2 ± 4.7[a]	33.0 ± 4[a]
GnRH (100 nM)	17.0 ± 1.6	86.0 ± 15[a]

Cultured rat pituitary cells were permeabilized by ATP^{4-} (6 μM) in the presence of EGTA (15 μM) and the absence of divalent cations for 5 min at 37 C. GTPγS was introduced during permeabilization and the cells were resealed by Mg^{2+} and further incubated with or without GnRH in the presence or absence of Ca^{2+}. Incubation was then continued for 2 h at 37 C, and LH was determined by RIA. [Reproduced with permission from R. Limor *et al.*: *Biochem Biophys Res Commun* 159:209, 1989 (119).]

[a] $P < 0.01$.

Ca^{2+}-independent (Table 1 and Ref. 119). Hence, gonadotropin secretion in permeabilized pituitary cells can be elicited by free Ca^{2+} buffers ($K_i = 0.3$ μM) or alternatively by GTP-γS in a Ca^{2+}-independent process. It is therefore possible that GTP activation of a G-protein will increase the affinity of the exocytotic response to Ca^{2+}. As activation of phosphoinositide turnover by GTP-γS but not by GnRH is Ca^{2+}-dependent (119, 128), GTP-γS-induced LH release might also be mediated via interaction with additional G proteins that are not coupled to PLC. This is also supported by findings that there is no direct correlation between the ability of a given nucleoside triphosphate to stimulate PLC activity and LH release in permeabilized pituitary cells. Interestingly, Cockcroft *et al.* (106) have recently suggested the existence of two G proteins (G_p and G_E) that are involved in stimulus-secretion coupling mechanisms in mast cells. Whereas G_p mediates its actions via enhanced phosphoinositide turnover, G_E acts at a more distal site and is not sensitive to neomycin inhibition (106). It is therefore possible that GTP-γS-induced gonadotropin secretion observed in our study was mediated also by a G_E-like protein. Similar results were observed in rabbit neutrophils permeabilized by sendai virus where secretion of β-glucuronidase was induced by guanine nucleotides in a Ca^{2+}-independent manner under conditions that did not enable activation of PLC (237, 238).

Small mol wt GTP-binding proteins were recently described (103, 239, 240). Among them are Yptlp and Sec4p from yeast, which are involved in secretory responses (103). In addition a *ras*-related G protein ($G_{22}K$) was isolated from human neutrophil membranes (240). $G_{22}K$ seems to be the substrate for ADP ribosylation by botulinum toxin type D that has been reported to inhibit secretion in various cell types (241). Therefore $G_{22}K$ might be similar or identical to G_E and might be involved in exocytotic responses. Whether a G_E-like protein is involved in GnRH action is not yet known. However, even if a G_E-like protein is involved in GnRH action it will be in addition to G_p, as GnRH action is tightly coupled to enhanced phosphoinositide turnover, which is responsible for Ca^{2+} mobilization and PKC activation in the gonadotrophs.

The results concerning the role of an intracellular Ca^{2+} pool in GnRH action do not agree with proposals that the Ca^{2+} required for GnRH-induced LH release is derived only from extracellular pools (9–11). In addition Huckle and Conn (242) found no increase in IP_3 formation by GnRH and inhibition of GnRH-induced Ins-P production, but not LH release, by TPA. This pharmacological uncoupling led the authors to suggest that GnRH-induced LH release is not dependent on the full expression of the PI response. On the other hand other groups found rapid elevation of IP_3 by GnRH (128, 181, 182) and mobilization of intracellular Ca^{2+} stores (17, 183, 243, 244). Moreover, no inhibition of GnRH-induced Ins-P formation by low concentrations of TPA was observed; hence uncoupling of PI turnover and LH release was not apparent (41, 128).

The second Ca^{2+} pool mobilized by GnRH is derived from extracellular sources via voltage-sensitive Ca^{2+}

channels (17, 223). This Ca^{2+} pool is not involved in the first burst phase of the exocytotic LH response to GnRH but takes part in the sustained phase of GnRH-induced LH release (17, 143, 223). The findings that dihydropyridine Ca^{2+} channel agonists and antagonists enhanced or inhibited GnRH-stimulated LH release further support the notion that voltage-sensitive Ca^{2+} channels (VSCC) are present in pituitary gonadotrophs and participate in GnRH action. More recently, using the Fura-2 method and single pituitary gonadotrophs identified by reverse hemolytic plaque assay, Shangold *et al.* (244) reported the changes in $[Ca^{2+}]_i$ induced by GnRH. A rapid rise was followed by a secondary more extended rise in $[Ca^{2+}]_i$. The responses frequently consisted of several rapid oscillations as reported in other systems (245, 246). The authors suggested that mobilization of Ca^{2+} might be mediated by IP_3 while Ca^{2+} influx might be induced by DG-activated PKC as addition of TPA mimicked the secondary phase of GnRH action on $[Ca^{2+}]_i$ (244). The secondary phase was blocked by nitrendipine, suggesting that Ca^{2+}-influx occurred via VSCC of the L-type (17, 244). However GnRH-induced depolarization in the gonadotrophs was not demonstrated in electrophysiological studies (247, 248). On the other hand, GnRH-induced voltage fluctuations resulted from the opening of Ca^{2+}-permeable channels (247, 248).

Recent observations demonstrated the existence of three types of Ca^{2+} channels (L, T, and N channels) only one of which (L-type) is susceptible to the dihydropyridine Ca^{2+} channel antagonists (249). As N channels are found in neurons it is still possible that in addition to the L-type (long) Ca^{2+} channels, the T-type (transient) channels are also involved in GnRH action. The presence of tetrodotoxin-sensitive Na^+ current and L- and T-type Ca^{2+} currents were found in rat pituitary corticotrophs (250). Two Ca^{2+} currents were also reported in tumor pituitary cells, lactotrophs and pars intermedia cells (251–254). The identification of gonadotroph Ca^{2+} currents awaits further investigation.

The third mechanism by which Ca^{2+} is mobilized by GnRH is by influx via voltage-insensitive channels. The mechanism involved in this mobilization and the relative contribution of this Ca^{2+} pool to GnRH action are still not clear.

Monitoring $[Ca^{2+}]_i$ in cell populations by the Quin-2 method represents an average of changes occurring at the level of the single cell. Indeed a discrepancy was found when changes in $[Ca^{2+}]_i$ were monitored by Quin-2 or Fura-2 in single GH_3 cells stimulated by TRH (255, 256). At this stage it is not clear whether a certain threshold in $[Ca^{2+}]_i$ must be reached in order for a ligand to exert its biological action as discussed in the Quin-2 studies (229, 230), or whether increased frequency of rapid $[Ca^{2+}]_i$ transients is more important as suggested in Fura-2 studies (245, 246).

Detailed analysis of the biphasic Ca^{2+} transients induced by TRH in GH_3 cells revealed a complex network of events participating in the process (227, 228, 256–258). The rapid first phase of Ca^{2+} mobilization from intracellular pools seems to be mediated not only by IP_3 but consists also of a PKC-sensitive mechanism. Furthermore, the Ca^{2+} transient is associated with an outward K^+ current resulting in hyperpolarization of the membrane which might reduce Ca^{2+} influx via VSCC as a mechanism of negative feedback (257). The PKC role in TRH-induced Ca^{2+}-transients in GH_3 cells is not clear. Drummond (259) suggested that PKC might subserve a negative feedback control on TRH-induced Ca^{2+} mobilization, and therefore be responsible for the descending part of the Ca^{2+} transient. On the other hand Mollard *et al.* (258) suggested recently that TRH-induced Ca^{2+} transient in GH_3B_6 cells is composed of two positive inputs that are mediated by IP_3 and PKC. Also, addition of Sn-1-oleoyl-2-acetylglycerol to GH_4C_1 cells induced a Ca^{2+} transient possibly by activation of VSCC (260).

The relative contribution of Ca^{2+} mobilization and influx to the process of gonadotropin secretion was evaluated by a pharmacological reconstitution experiment (17). Since ionomycin will initially mobilize cellular Ca^{2+} (Fig. 4) and will later also cause Ca^{2+} influx, it is a good tool with which to analyze the contribution of Ca^{2+} (intra- and extracellular pools) to the exocytotic response. LH release by a concentration of ionomycin capable of elevating $[Ca^{2+}]_i$ to that induced by GnRH represented only about 45% of the GnRH response (Fig. 6). Therefore Ca^{2+} is necessary but not sufficient to mediate GnRH-induced gonadotropin secretion.

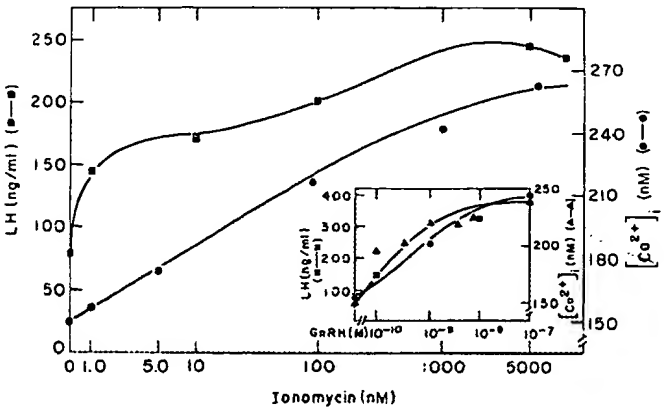

FIG. 6. Effect of the Ca^{2+} ionophore ionomycin and GnRH (*inset*) on $[Ca^{2+}]_i$ and LH release in pituitary gonadotrophs. Cells were loaded with Quin 2/AM and stimulated with increasing doses of ionomycin or GnRH. A separate batch of cells was stimulated with ionomycin and GnRH for 2 h and LH release to the medium was measured by RIA. [Reproduced with permission from Z. Naor *et al.*: *Mol Endocrinol* 2:512, 1988 (17). © The Endocrine Society.]

VI. PKC

Since its discovery the Ca^{2+} and phospholipid-dependent PKC has gained recognition as a key regulatory element in the network of cellular signalling (135, 136). PKC is now believed to be involved in diverse processes such as synaptic transmission, neuronal development, axonal regeneration, exocytosis, growth, differentiation, learning, and tumor promotion. The discovery that PKC is the main receptor for the tumor promoter phorbol esters implicated PKC in the process of transformation and also suggested involvement of the enzyme in the action of certain oncogenes (*e.g. sis, ras, erb* B) (135, 136, 261, 262).

PKC is activated by association with membrane phospholipids, in particular phosphatidylserine (PS) in the presence of elevated Ca^{2+}. Unsaturated DG increases the apparent affinity of PKC for PS and Ca^{2+}, enabling activation of the enzyme at physiological $[Ca^{2+}]_i$. Since DG is generated during enhanced phosphoinositide turnover, PKC is therefore involved in signal transduction mechanisms of Ca^{2+}-mobilizing receptors, which operate via PI turnover. It is assumed that IP_3-induced Ca^{2+} mobilization will expose hydrophobic groups in the PKC molecule that will attach the enzyme to the membrane DG moieties, which serve as anchoring units (by means of hydrophobic interaction and H-bonds), and enable binding of the enzyme to 4 molecules of PS, which are present in abundance in membrane phospholipids. Also, perturbation of the bilayer structure by DG might induce a phase transition enabling the hydrophobic regulatory domain of PKC (30 kDa) to penetrate the hydrocarbon part of the bilayer while the hydrophilic catalytic domain [50 kDa, protein kinase M (PKM)] will face the cytosol. Translocation of PKC to the membrane is regarded as the principle mechanism of its activation (263).

The nature of the PKC substrate plays an important role in imparting the cofactor requirements for PKC activation (264). Whereas protamine for example is phosphorylated in the absence of Ca^{2+}, PS, and DG, myelin basic proteins require the presence of phospholipid alone, while histone and troponin require the presence of all three cofactors (264).

Although DG is thought to be the natural activator of PKC, a recent report suggested that PIP_2 may precede DG in activating the enzyme (265). When PKC activity was measured by mixed micellar assay with Triton X-100 according to Hannun *et al.* (266), Chauhan and coworkers found that the K_{app} for PIP_2 was about 0.04 mol % while that for DG was about 2 mol %. The fact that PIP_2 represents about 0.2 mol % of total membrane lipids makes it a good candidate for PKC activation since DG concentration in the membrane as a breakdown product of PIP_2 must be less than 0.2 mol % and hence not sufficient to reach the 2 mol % required. (The calculations assume that DG is derived only from PIP_2, which might not be the only source as discussed above). It was therefore suggested that DG is needed to bind or translocate the enzyme to the membrane, whereas both DG and PIP_2 will then activate PKC (265).

PKC was regarded as a single monomeric entity; however analysis of cDNA clones of the enzyme revealed a multiple gene family encoding at least 7 subspecies (135, 267–281). The first 4 to be discovered (γ, βI, βII, and α) all consist of a single polypeptide chain with 4 conserved (C_1–C_4) and 5 variable (V_1–V_5) regions. Common to the 4 subspecies is their sensitivity to activation by Ca^{2+}, PS, and DG, albeit to variable extent (135, 282–286). The 3 new subspecies designated δ, ϵ, and ζ lack the C_2 region and show no absolute requirement for Ca^{2+}, PS, and DG, suggesting that the conserved C_2 region is involved in the cofactor binding and activation process (135, 278, 281). Another subspecies of PKC (nPKC) was recently identified in rabbit brain (287), and it seems to correspond to the ϵ-subspecies (281). Members of the *raf* oncogene family such as c-*raf* and A-*raf* show structural similarity to the PKC family and contain a serine/threonine protein kinase domain and therefore might represent additional members of the PKC or related families (288, 289).

The catalytic domain containing the C_3 and C_4 regions is similar among the various subspecies and contains the ATP-binding domain. The V_3 region contains a cleavage site that can be activated by calpain resulting in the release of the 50 kDa catalytic domain (PKM), which is active in the absence of Ca^{2+}, PS, and DG (290, 291). According to some investigators this pathway represents a physiological activation process (292). If this is the case, the physiological PKC substrates might be found also in the cytosol. On the other hand, if translocation to the membrane represents the physiological activation process, and the formation of PKM is only a mechanism of inactivation, one would expect that membrane proteins will serve as substrates for the enzyme. This issue has not yet been clarified.

Using biochemical, immunohistochemical, Northern blot analysis, and *in situ* hybridization methods, several groups demonstrated differential expression of the PKC subspecies among brain areas, tissues, cells, and cell lines (135, 275, 279, 280, 293–300). It is thought now that the γ-subspecies is present only in central nervous tissues, the α-subspecies in all tissues and cell lines examined, and the β-subspecies is present in most tissues examined. The subspecies might therefore specialize in specific functions. When rat cerebellar cortex was stained with type-specific antibodies, Purkinje cell bodies reacted with the γ-antibodies, the molecular layer which is rich in presynaptic nerve endings was stained by the βII-anti-

bodies, while the granular layer stained with the βI-antibodies (298). The above findings strengthen the notion of specific functions for the various subspecies which might be achieved in different ways: 1) different subspecies might be activated similarly; however the presence of a specific subspecies in a given cell type will dictate the response; 2) different subspecies might be activated similarly and even be present in the same cell; however each of them phosphorylates different substrates; 3) similar to 2) but each of the subspecies displays a different degradation rate (291, 301, 302); 4) different subspecies might be activated differentially by various second messengers and phosphorylate similar or different substrate proteins (285, 286, 303).

Characterization of the kinetic and catalytic properties of the various subspecies revealed differential responses to second messengers. The α- and, in particular, the γ-subspecies showed much less response to DG or TPA activation as compared to the β-enzyme which was highly responsive (282–285). In addition the three new subspecies, δ, ϵ, and ζ, show little responsiveness to Ca^{2+} and DG (281).

It is possible that enhanced phosphoinositide turnover will result in selective activation of γ, βI, βII, and α-PKC according to elevation of $[Ca^{2+}]_i$ and the half-life of DG in a given cell system. On the other hand production of DG in the absence of elevated $[Ca^{2+}]_i$, as is the case with PLC or PLD acting on phosphatidylcholine or hydrolysis of PI-Glycans, might activate the new PKC subspecies (δ, ϵ, ζ) as PKC-ϵ was shown to be activated in a Ca^{2+}-independent fashion.

Surprisingly, when the brain subspecies (α, β, γ) were activated by low concentrations of AA (12 μm), only the γ-subspecies was markedly activated even in the absence of Ca^{2+}, PS, and DG (Fig. 7 and Refs. 285 and 286). The α-subspecies could also be activated by the fatty acid, albeit this activation process required Ca^{2+} and much higher concentration of the fatty acid (282). Among several AA metabolites that were tested, Lipoxin A markedly activated the γ-enzyme in the absence of Ca^{2+}, PS, and DG (286). It is therefore possible that PI turnover represents only one biochemical pathway capable of activating PKC subspecies whereas other mechanisms elicited by phospholipase A_2 and PLD (187–193) deserve further investigation.

A. PKC subspecies in the hypothalamo-pituitary axis

Analysis of PKC subspecies revealed the presence of γ, βI, βII, and α in the rat hypothalamus, while the anterior or posterior pituitary contains only the βII- and α-PKC subspecies (Table 2 and Ref. 285). Other reproductive endocrine glands such as testis, ovary, and placenta contain also only the β- and the α- but not the γ-subspecies. However, whereas the pituitary lacks measurable amounts of βI, the testis, ovary, and placenta express the βI-enzyme, albeit to a much less extent as compared to the βII-enzyme (Z. Naor, in preparation). In terms of specific activity, pituitary PKC is only about 40% active compared to the hypothalamic enzyme and about 10% compared to whole brain PKC. The β- and α-subspecies of the hypothalamus and the pituitary are recovered from the soluble (60%) and particulate (40%) fractions, whereas the hypothalamic γ-PKC was found only in the soluble fraction (285). Western blot analysis with type-specific antibodies confirmed that the hypothalamic and the pituitary enzymes that were eluted from the HPLC-hydroxyapatite columns are immunologically similar to the brain subspecies (285). The hypothalamic γ-enzyme is relatively resistant to activation by TPA or DG and is highly responsive to AA in the absence of the cofactors (Fig. 7 and Ref. 285). The α- and β-PKC of both hypothalamus and the pituitary are sensitive to TPA or DG stimulation, with the hypothalamic subspecies being less responsive. The pituitary subspecies displayed higher affinities for phorbol ester binding as com-

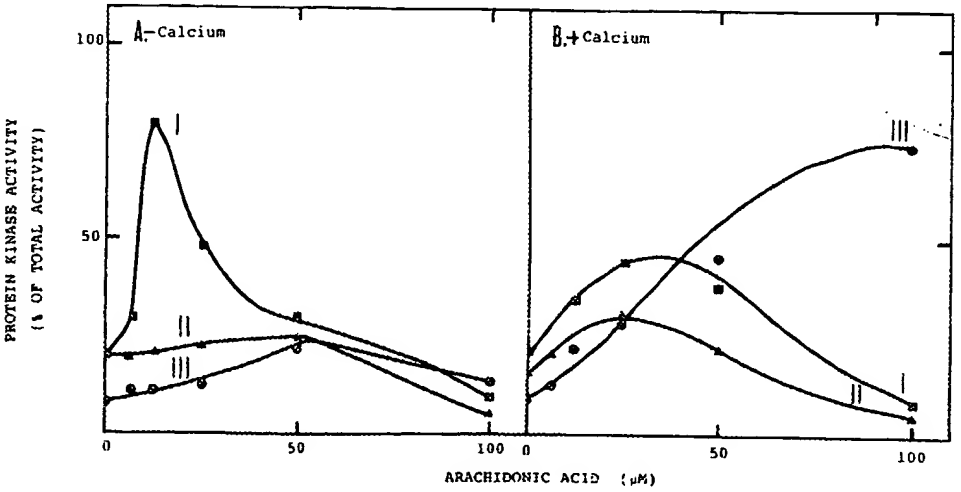

FIG. 7. Dose-response study of AA-stimulated PKC activity of the hypothalamic subspecies in the presence and absence of Ca^{2+}. Hypothalamic PKC subspecies (from 30 rats) were prepared as described in Ref. 285. Samples (20 μl each) were assayed in the absence of Ca^{2+} and presence of EGTA (0.5 mM) (A) or in the presence of 0.3 mM Ca^{2+} (B). PS and DG were replaced by various concentrations of AA. Results are normalized to Ca^{2+}, PS, and DG activation. [Reproduced with permission from Z. Naor *et al.*: *Mol Endocrinol* 2:1043, 1988 (285). © The Endocrine Society.]

TABLE 2. Relative distribution of PKC subspecies in the hypothalamus and pituitary

Enzyme subspecies	PKC activity (%)				PKC Immunoactivity (% of soluble enzyme)	
	Hypothalamus		Pituitary		Hypothalamus	Pituitary
	Soluble	Particulate	Soluble	Particulate		
Type I (γ)	24 ± 4.5	4 ± 1.2	ND	ND	13 ± 2.5	ND
Type II	42 ± 3.0	54 ± 6.5	42 ± 6.0	39 ± 5.0	36 ± 4.0	41 ± 6
(βI)					24 ± 1.5	ND
(βII)					76 ± 5.0	100
Type III (α)	34 ± 3.5	42 ± 5.5	58 ± 5.5	61 ± 10	51 ± 3.5	59 ± 8

Soluble and particulate enzyme preparations from 30 rat hypothalami or 160 rat pituitaries were prepared, and total fraction activity was obtained from the individual fractions of the peaks. Blots obtained with a monoclonal antibody against rat brain PKC and with peptide antibodies for the βI and βII subspecies were analyzed by densitometric tracing to obtain the immunoreactivity data. ND, not determined. [Reproduced with permission from Z. Naor: *Endocrinology* 126:000, 1990 (320). © The Endocrine Society.]

pared to the respective hypothalamic counterparts. Whether the new members of the PKC family (δ, ϵ, and ζ) are also present in the hypothalamus and the pituitary is not yet known. The heterogeneity of subspecies expression and responsiveness suggest specific roles for the various subspecies in the hypothalamo-pituitary axis.

B. Role of PKC in GnRH action

Early and more recent observations that TPA and synthetic DGs stimulate LH release were interpreted as indicating a role for PKC in GnRH-induced gonadotropin secretion (58, 171, 304–307). Also pituitary PKC (308) was shown to be redistributed by GnRH from the cytosol to the membrane *in vivo* (52, 53) and *in vitro* (51). Since redistribution of the enzyme is the main mechanism of its activation, PKC was implicated in GnRH-induced gonadotropin secretion (51–53). Since TPA can mimic only partially the GnRH response, and the simultaneous presence of Ca^{2+} ionophore and TPA produced a synergistic effect, it was suggested that activation of the bifurcating Ca^{2+} messenger system, namely elevation of $[Ca^{2+}]_i$ and activation of PKC is necessary for eliciting the full GnRH response (304).

More recently it was proposed that PKC is not involved in GnRH-induced gonadotropin secretion, since the neurohormone action was not impaired in PKC-depleted rat pituitary cells (309). Further support to this claim emerged from observations in ovine pituitary cells where it was noted that the stimulatory effects of GnRH and TPA were additive (310). Also, Johnson *et al.* (311) found no inhibition of GnRH action by the PKC inhibitors H-7 and polymyxin B. Nevertheless, our data (312) and that of Stojilkovic *et al.* (313) strongly implicated PKC in GnRH action. Using prolonged incubation protocols with TPA to down-regulate endogenous PKC, Stojilkovic *et al.* (313) found impairment in GnRH-induced gonadotropin secretion. The discrepancy (Ref. 309 *vs.* Ref. 313) may be explained by recent observations in our laboratory that data presented as percent of LH content do not show consistency as compared to data presented as absolute amount of LH released from PKC-depleted cells (312). Indeed, we also found a reduction in GnRH response in terms of LH release in PKC-depleted cells when the data were presented in absolute values (312). This method of data presentation is justified as the nature of the response does not depend on the size of the LH pool as evident from observations that ionomycin presented to normal or to TPA-pretreated cells elicited a similar response (312). Unlike Beggs and Miller *et al.* (310), who reported additivity between GnRH and TPA in ovine pituitary cells, we (41) and others (313) found no additive effect on gonadotropin secretion in rat pituitary cells. The discrepancy might be due to a relatively low response of ovine pituitary cells to the GnRH stimulus as compared to rat cells. Also staurosporine, a relatively selective PKC inhibitor which acts in the nanomolar range, was recently found to inhibit the stimulatory effect of both GnRH and TPA upon gonadotropin secretion with a similar IC_{50} of about 80 nM (312). Therefore inhibition of GnRH action by PKC depletion and staurosporine inhibition suggest a role for the enzyme in the exocytotic response of GnRH. Support for involvement of PKC in GnRH action comes also from observations that similar phosphoprotein substrates were identified for GnRH and TPA in cultured pituitary cells, and phosphorylation of the GnRH substrates was inhibited in PKC-depleted cells (314).

The discrepancies concerning the role of PKC in GnRH-induced gonadotropin secretion (309–314) might result from TPA-induced PKM formation, which might have participated in GnRH action. Interestingly, addition of PKM to neutrophil membranes activated NADPH-oxidase, the enzyme responsive for oxidative burst in neutrophils, similar to the effect of TPA on the intact cells (292). In addition, it is not clear whether TPA down-regulates all PKC subspecies. The fate of the various PKC subspecies after TPA pretreatment was

recently investigated in several cell systems (302, 315–317). Treatment of human platelets or U937 promyelocytic leukemia cells with TPA resulted in redistribution and down-regulation of PKC-α, whereas the β-enzyme remained cytosolic and was not degraded (302, 315). On the other hand, TPA-treated RBL-2H3 cells, or TPA-treated KM3 cells showed rapid down-regulation of β-PKC, whereas α-PKC was more resistant to the TPA treatment (316, 317). When purified enzymes were exposed to trypsin (316), or to calpain (291), type III (α-PKC) was found to be relatively resistant to proteolytic digestion, β-PKC was intermediate, and γ-PKC was rapidly degraded. The discrepancy outlined above is not yet clear but might have to do with the specificity of the peptide-antibodies used by the various groups. In any case it is clear that the paradigm of PKC down-regulation by prolonged TPA pretreatment should be reexamined and evaluated separately for each subspecies of PKC.

It is therefore possible that pituitary gonadotrophs contain a TPA-insensitive subspecies that has not yet been characterized and whose role in GnRH action cannot yet be determined. Interestingly, additional cDNA clones for PKC were recently described (δ, ϵ, ζ) that lack the C_2, which is part of the regulatory domain, and they show little responsiveness to Ca^{2+} and DG (278, 281). So far we have identified the presence of α and βII in the anterior pituitary but not the γ-PKC which is most likely present only in brain and spinal cord (135). Whether additional members of the PKC family are present in the pituitary is still not known.

Since it is difficult to overexpress a given PKC subspecies in primary cultures of pituitary cells using cDNA transfection techniques, we have recently developed a method of "depletion-insertion" for PKC subspecies (318). In this method we first down-regulate endogenous PKC activity by prolonged incubation with TPA, followed by digitonin permeabilization and insertion of the purified brain subspecies (Fig. 8). Interestingly, digitonin permeabilization by itself results in loss of endogenous PKC activity, most likely due to release of PKC to the medium (318, 319). Thus the combination of TPA pretreatment and digitonin permeabilization provides a suitable background with which to analyze the biological effect of specific subspecies. Utilizing the depletion-insertion method we found that insertion of activated α- and β- but not γ-PKC to TPA-pretreated, digitonin-permeabilized rat pituitary cells resulted in LH release (Fig. 8 and Ref. 318). Both α- and β-PKC are present in the anterior pituitary (285, 320) and therefore might be utilized by GnRH during the course of exocytosis. The above "pharmacological reconstitution" approach implicated PKC in the exocytotic response elicited by GnRH. Since γ-PKC is present only in brain and spinal cord tissues and had no effect on LH release, one would argue

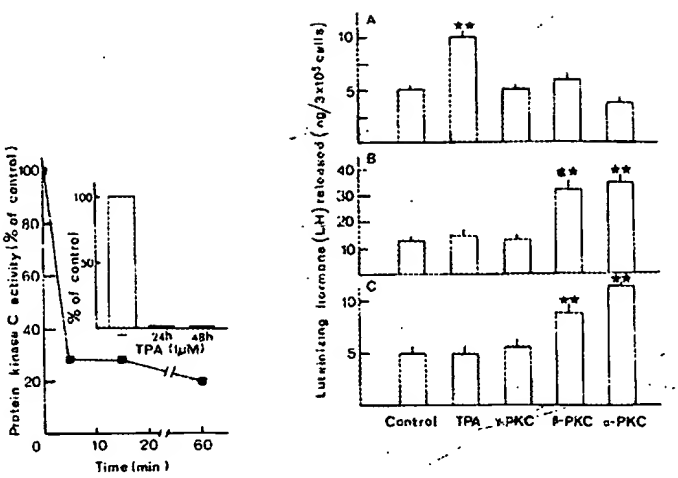

FIG. 8. Insertion of brain PKC subspecies to permeabilized down-regulated pituitary cells. Loss of PKC activity in pituitary cells permeabilized by digitonin or incubated with 1 μM TPA (*inset*, left panel). Anterior pituitary cells (10^7 per tube) were permeabilized by 16 μM digitonin for the time indicated or cultured for 24–48 h with 1 μM TPA. Cells were then homogenized, and total PKC activity was determined after 0.5% Triton X-100 extraction and DE-52 chromatography. Basal activity (100%) amounted to 2.7 pmol $^{32}P/\mu g$ of protein·min. PKC subspecies were prepared from adult male rat brains as described in Ref. 318. For insertion studies the cultured cells (3×10^5 per dish) were treated with 1 μM TPA at 37 C (C) or without TPA for 24 h (A and B). The cultured cells were then left intact (A) or permeabilized by 16 μM digitonin treatment for 5 min (B and C) at 37 C in high-K^+ buffer. The cultured permeabilized cells were then washed and further incubated in glutamate buffer. The normal cells (A) were incubated in medium 199 containing 0.1% BSA. The stimulants were TPA (100 ng/ml) and PKC subspecies (2.5 μl; ~60 ng enzyme activated by TPA at 100 ng/ml). Incubation was continued for 30 min at 37 C, and medium was then collected and stored at −20 C until assayed for LH by RIA. **, $P < 0.01$. [Reproduced with permission from Z. Naor *et al.*: *Proc Natl Acad Sci USA* 86:4501, 1989 (318).]

that the γ-enzyme is a "brain specific PKC" and specializes only in neuronal functions. However when NIH3T3 cells were transfected with γ-PKC cDNA, enhanced tumorigenicity was found (321). Nevertheless, the effect observed was much less pronounced compared to *ras*-transformed cells, suggesting that tumorigenicity is not the main task of γ-PKC.

In addition exposure of HL-60 cells to dimethyl-sulfoxide or retinoic acid, and exposure of U937 promyelocytic leukemia cells to TPA, induced the appearance of γ-PKC which was not detected in untreated cells (302, 322). Although γ-PKC was not described in extra central nervous system tissues and cells, it is possible that under certain conditions some cell types will express the enzyme during differentiation.

Several cell lines express only the α-PKC; hence the enzyme was implicated in growth and differentiation (303). It was therefore interesting to find that α-PKC could elicit an exocytotic response in the permeabilized pituitary cells (318).

Overproduction of PKC subspecies by transfection

experiments (321, 322) and the insertion studies described above suggest that physiological or pathological functions mediated by PKC might be executed by more than one subspecies. If this is the case then specificity of subspecies activation might also be achieved by alternative biochemical pathways. As mentioned above, the γ-PKC can be activated by Ca^{2+}, PS, and DG or alternatively by low concentrations of AA in the absence of the above cofactors (285, 286). Future studies will concentrate on the characterization of pituitary PKC subspecies and their relative roles in GnRH action.

VII. AA and Its Metabolites

AA is released from the 2-acyl position of membrane phospholipids (323). Various phospholipases can bring about the release of AA: direct hydrolysis by phospholipase A_2 or indirectly by PLC followed by DG- and monoacylglycerol lipase. Another route involves activation of phospholipase A_1 followed by lysophospholipase (phospholipase B) (323, 324). Finally, PLD followed by PA phosphohydrolase, DG-, and monoacylglycerol lipase will also generate AA. The free fatty acid is rapidly metabolized to eicosanoids (prostaglandins, thromboxane, and leukotrienes) or else reesterified by acyl transferases back to phospholipids (323, 325). The high turnover rate of AA ensures an off mechanism to lower the cellular concentration of the fatty acid and hence makes it a good candidate for a second messenger in analogy with IP_3 and DG. Since AA formation is coupled in some cases to the formation of IP_3 and DG (*e.g.* phosphoinositide turnover), Ca^{2+}-mobilizing ligands might utilize the services of AA as a messenger molecule. Formation of various second messengers is coupled to a G protein (*e.g.* cAMP, IP_3, DG), and it was therefore interesting to note that phospholipase A_2 might be operating via a pertussis toxin-sensitive G protein that differs from G_p (108, 326). Also, common to second messengers is the activation of a protein kinase. Indeed α-PKC is activated by high concentrations of AA in the presence of Ca^{2+}, and it is this activation process that was most likely observed in early reports on PKC activation by fatty acids (282, 284, 327–330). More interesting, however, is the activation of γ-PKC which is observed at very low, possibly physiological concentrations of AA (~10 μM) in the absence of Ca^{2+}, PS, and DG (285, 286). Among AA metabolites tested, only lipoxin A [5(S),6(R),15(S)-11-cis-isomer] and 12(s)-hydroxy-5,8,10,14-eicosatetraenoic acid were active in γ-PKC activation (286). As γ-PKC is relatively resistant to the DG and TPA activation (285, 286), it is possible that under certain conditions the activation process of γ-PKC might be dissociated from phosphoinositide turnover. Such a mechanism will broaden the range of potential hormones capable of activating PKC and will prolong the enzyme activation in the face of vanishing levels of DG.

Other lines of evidence also support a role for AA as a potential second messenger. AA and its lipoxygenase products were implicated in activation of myocytes and Aplysia K^+ channels (113, 114, 116, 117). Application of AA into the hippocampus enhanced long term potentiation (331). Arachidonate was reported to activate PLC, adenylate, and guanylate cyclase and to mimic the action of IP_3 on cellular Ca^{2+} mobilization (332–336). Finally, AA was reported to stimulate exocytosis in the pituitary (171, 232–236, 335, 337), placenta (332), and pancreas cells (338).

GnRH was shown to stimulate AA formation (171), but the phospholipase involved has not yet been identified. Prostaglandins are not involved in GnRH-induced gonadotropin secretion (339, 340) or GnRH-induced progesterone and testosterone production at the gonadal level (341, 342). On the other hand AA and its lipoxygenase or epoxygenase derivatives might be involved in the exocytotic response of GnRH (232–236). This is based on the observations that AA and some metabolites stimulate LH release, and lipoxygenase inhibitors block GnRH-induced gonadotropin secretion. In a separate study it was shown that enriched pituitary gonadotrophs display lipoxygenase activity (231). AA and its lipoxygenase products were also implicated in PRL (335, 337, 343, 344), TSH (345, 346), and insulin release (347, 348).

The molecular mechanism of AA action is not known. Since γ-PKC is not present in the pituitary or pancreas it is unlikely that the effect was exerted by direct activation of PKC. It is possible that AA was acting by means of Ca^{2+} mobilization (334). Alternatively, AA can be converted to a leukotriene which exits the cell and binds to its own receptor, activates phosphoinositide turnover, and possibly PKC, and thus propagates the ligand signal (349). Therefore studies are needed to support a role for AA and its metabolites as first or second messengers.

VIII. Gonadotropin Biosynthesis

The effect of GnRH on gonadotropin biosynthesis has gained momentum with the availability of cDNAs for the gonadotropin subunits (350–357). Earlier studies utilized labeled amino acids and glucose amine, but the signal-noise ratio was low and it was not well clarified whether GnRH affects transcription, translation, or postranslation modification such as glycosylation (202–204, 358–368). Nevertheless, increase in rat LH polypeptide chain synthesis by GnRH was demonstrated when [^{35}S]methionine labeling and immunoprecipitation techniques were used (204, 368). The effect was noticed only after a lag (~1–2 h) while LH release was immediate (368).

Endogenous GnRH is required for gonadotropin gene expression as demonstrated in castrated rats, mice, and ewes where castration elevates α and LHβ mRNA levels, whereas removal of endogenous GnRH restores precastration values (369–372). The postcastration rise in α and LHβ mRNAs can also be obliterated by steroid replacement exerting negative feedback (373–375). The direct positive effect of estrogen at the pituitary level may occur at the level of the LHβ gene since a 5′-flanking region of the gene was found to bind estrogen receptor and to confer hormonal responsiveness to an heterologous promoter (376). GnRH pulse injections to castrated-testosterone-replaced male rats, or to female hypogonadal (hpg) mice, increased both α and LHβ mRNA levels albeit to a different extent and with different kinetics (374, 377, 378). Maximal stimulation of LH gene expression is only seen over a narrow range of GnRH pulse amplitude and frequency (374). LHβ mRNA levels may be a limiting factor in GnRH action as might have been expected since α is common to LH, FSH, and TSH.

Indeed during desensitization induced by continuous GnRH administration to male rats, LHβ mRNA levels decreased while α-mRNA increased, suggesting that LHβ is more tightly coupled to GnRH action (379). The data might also explain the paradoxical finding in patients treated with GnRH agonists in whom high plasma levels of free α-subunit were found, although the pituitary was desensitized.

In vitro experiments utilizing primary pituitary cultures are more difficult to interpret since controversy exists concerning the regulation of LHβ mRNA by GnRH. Long term incubation of cultured pituitary cells with low doses of GnRH increased α-mRNA levels with no change in LHβ mRNA (380). Similarly, GnRH stimulation of LH release *in vitro* does not appear to be coupled to increased rate of LHβ gene transcription or to cytoplasmic mRNA levels (381). In contrast, it was reported that GnRH increased LHβ mRNA levels by a PKC-mediated mechanism (382).

Summary

Multiple (at least seven) steps are involved in GnRH-induced gonadotropin secretion and gonadotropin gene expression (Fig. 9). After binding to specific receptors located exclusively on pituitary gonadotrophs, GnRH stimulates a rapid phosphodiesteric hydrolysis of phosphoinositides for which no rise in $[Ca^{2+}]_i$ is required. Activation of PLC is most likely mediated by a pertussis toxin-insensitive GTP-binding protein (G_p). In its activated state (G_p-GTP) the binding affinity of GnRH to is receptor is reduced. Rapid formation of IP_3 will enhance Ca^{2+} release from intracellular sources most likely via a specific IP_3 receptor. The transient Ca^{2+} rise might be

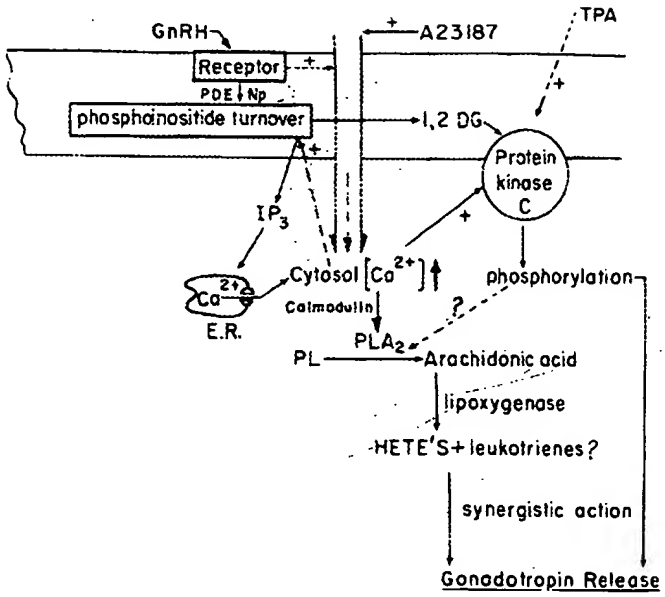

FIG. 9. Proposed model for GnRH action on gonadotropin secretion. PDE, Phosphodiesterase representing PLC; N_p or G_p is a GTP-binding protein; E.R., endoplasmic reticulum; PL, phospholipids, PLA_2, phospholipase A_2; HETE, hydroxyeicosatetraenoic acid. It is not yet clear whether AA participates in GnRH action in the "down stream" part as shown here, or at the "up stream" part as shown in Fig. 2.

responsible for a burst phase of LH release lasting for about 100 sec, which is not dependent on extracellular Ca^{2+}. The backbone moiety of the phosphoinositides, DG, and the elevated $[Ca^{2+}]_i$ are most likely responsible for translocation of PKC subspecies from the cytosol to the membrane. The most likely candidates are α- and βII-PKC. The activated PKC subspecies phosphorylate substrate proteins which activate secretory reactions and participate in gonadotropin gene expression. In parallel Ca^{2+}-influx via nifedipine-sensitive and insensitive channels further elevates $[Ca^{2+}]_i$, which participates in the sustained phase of gonadotropin secretion in concert with the activated PKCs. GnRH also triggers the release of AA and the formation of lipoxygenase and/or epoxygenase products of the fatty acid which are also involved in the process of the exocytosis. We predict that the continuous supply of DG and AA needed for GnRH action is also provided via activated PLD which will also supply phosphatidic acid, the role of which is as yet unclear. The interaction of the various second messengers involved in GnRH action (IP_3, Ca^{2+}, DG, AA) and their relative roles in gonadotropin secretion and gonadotropin gene expression await further investigation.

In several aspects GnRH action on gonadotropin secretion is unique when compared to other Ca^{2+}-mobilizing ligands: 1) At physiological concentrations GnRH up-regulates its own receptors whereas most ligands down-regulate the respective receptor; 2) PKC up-regulates GnRH receptors whereas in most cases PKC down-

regulates the ligand receptor; 3) GnRH stimulation of PLC activity is most likely mediated by G_p whereas some Ca^{2+}-mobilizing ligands operate via G_i; 4) Activated PKC does not exert negative feedback upon GnRH-induced inositol phosphate production as is the case with several other peptides; 5) Activated PKC might be responsible for Ca^{2+} influx whereas in several other systems PKC is inhibitory to Ca^{2+} influx.

A large number of G protein-coupled receptors utilize a very small group of effector systems (adenylate cyclase, cGMP phosphodiesterase, phospholipase A_2, PLC, and PLD) in the course of signal-transduction. The systems involved include sensory transduction (vision, taste, and olfaction), synaptic transmission, growth, differentiation, exocytosis, and tumor promotion. One of the tasks of the signal-transduction cascade is to amplify the signal ($\sim\times10^5$) (111). The recent availability of cloned receptors (20, 21, 383, 384), G proteins (112), adenylate (385) and guanylate cyclase (194, 195, 386), PLC (145–148, 151), and the recently cloned IP_3 receptor (387) will improve our understanding of the nature of receptor-transducer-effector interaction in signal transduction (388).

When did vertebrate signal-transduction evolve? For one thing signal transduction mechanisms in prokaryotes and eukaryotes differ significantly. On the other hand similarity exists between signal transduction in eukaryotic microbes and vertebrate animals. It is therefore possible that vertebrate signal-transduction evolved with the origin of the early eukaryotes (389).

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